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Note

Susceptibility of Biofilm *Escherichia coli*, *Salmonella* Enteritidis and *Staphylococcus aureus* to Detergents and Sanitizers

SHIGEKO UEDA* AND YOSHIHIRO KUWABARA

Laboratory of Hygiene, Kagawa Nutrition University,
3-9-21, Chiyoda, Sakado-shi, Saitama 350-0214, Japan

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This study was conducted to investigate the susceptibility of the biofilm cells of *Escherichia coli* O157, *Salmonella* Enteritidis, and *Staphylococcus aureus* to some cleaning detergents and sanitizers. No weakly acidic, neutral, and weakly alkaline detergent could remove the biofilm bacteria from stainless steel chips at commonly used concentrations recommended by manufacturers. Among sanitizers, sodium hypochlorite did not completely inactivate any biofilm bacteria at active chlorine concentrations of 25 to 200 μg/ml. Benzalkonium chloride, alkyldiaminoethyl glycine hydrochloride, chlorhexidine digluconate, and polyhexamethylenebiganide inactivated the great majority of *E. coli* and *S.* Enteritidis at commonly used concentrations, but did not inactivate *S. aureus* effectively enough. The biofilm *S. aureus* population was shown to be more tolerant than the *E. coli* and/or *S.* Enteritidis populations to the sanitizers.

Key words : Biofilm/Enteropathogenic bacteria/Detergent/Sanitizer.

In the food-processing environment, biofilms are formed easily on the moist surfaces such as the counter, cutting board, utensils, and instruments so that there are a variety of nourishment sources including food residues for bacteria. A biofilm is defined as surface-attached communities that are composed of microorganisms and their extracellular polymeric substances. A cleaning and sanitation program in the food-processing environment is part of the process to inactivate the microorganisms and to prevent the accumulation of microbial cells, biofilms and particulates on the surface of utensils and equipment (Dunsmore et al., 1981). To provide consumers with wholesome and safe products, it is very important to control most of the microorganisms including enteropathogenic bacteria on processing surfaces as well as in food products (Pontefract, 1991). This study was undertaken to evaluate the efficacy of cleaning detergents and sanitizers commonly used in the food industry against biofilm enteropathogenic bacteria.

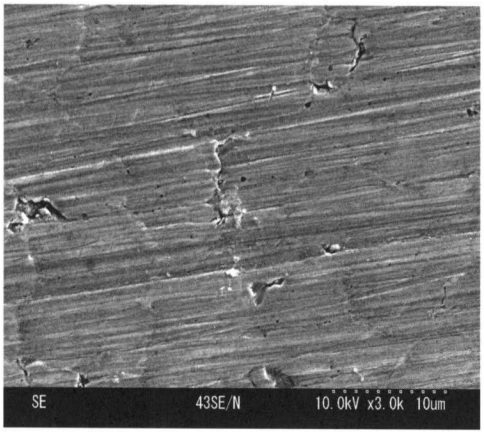
**Corresponding author. Tel/Fax : + 81-49-282-7343.*

The test organisms were *Escherichia coli* O157:H7 (verotoxin producer), *Salmonella* Enteritidis, and *Staphylococcus aureus* (A type enterotoxin producer), all of which have been isolated from food samples incriminated in food poisoning outbreaks. The cleaning detergents tested included a neutral detergent (containing 38% alkyl glucoside, pH6.72, Kao), a weakly acidic detergent (containing 43% polyethylene alkylether-fatty acid alkanol amide-alkyl ether sulfate, pH6.40, Lion), and a weakly alkaline detergent (containing alkyl ether sulfate-alkyl amine oxide, pH7.76, P & G). The sanitizers used the formulations of sodium hypochlorite (Wako), benzalkonium chloride (Sankyo), alkyldiaminoethyl glycine hydrochloride (Wako), chlorhexidine digluconate (Wako), and polyhexamethylenebiguanide hydrochloride (Rikokyosan).

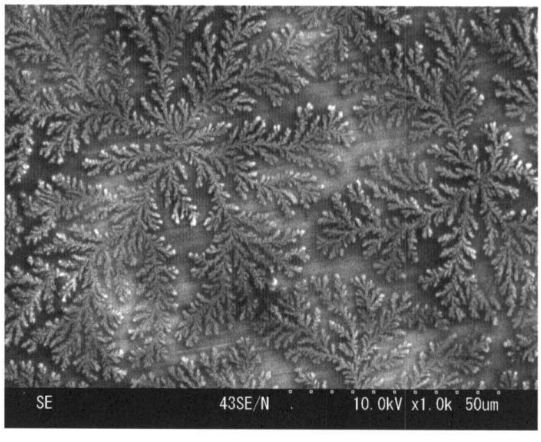
The biofilm bacteria were prepared according to the procedures of Ren and Frank (1993) and Peng et al. (2001). Briefly, the biofilm cells were prepared by using tryptic soy broth (TSB, BBL) as the growth

medium, and stainless steel chips (type 304, 2×2 cm) were used as a support. New stainless steel chips were submerged in a mixture of ethanol and acetone (1:1, v/v) and sonicated for 1h to remove grease. The chips were then rinsed with double-distilled water, immersed in 2% (w/v) NaOH solution for 5 min at 75℃, rinsed again with distilled water, immersed in 1% (v/v) HNO_3 for 5 min at 75℃, and then given a final rinse with distilled water. The prepared chips were placed in 25×180mm culture tubes with 25ml of TSB and sterilized by autoclaving. The medium was inoculated with 2ml from a TSB preculture of *E. coli* O157:H7, *S.* Enteritidis or *S.aureus* and incubated at 37℃ for 48h. The chips were then removed from the tube, rinsed three times by immersion in 25ml of sterile phosphate-buffered solution (PBS, 0.31mM KH_2PO_4) for 1min. After being washed, each chip was transferred to another tube containing 25ml of TSB and was incubated to develop the biofilm cells at 37℃ for 8d, during which period TSB was replaced every 2d with fresh sterile TSB to leave only the adhering cells and to provide fresh nutrients. After incubation, the chips were aseptically removed and rinsed three times (1 min each time) by immersion in sterile PBS to remove unadhering cells. It was confirmed that the biofilm cells adhered well to the stainless steel chips by scanning electron microscopic examination (Fig. 1). They were then used immediately in experiments as biofilm bacteria. The prepared biofilm chips were submerged in 40ml of different concentrations of detergents or sanitizer solutions for 5 min. The solution was continuously stirred throughout the exposure. At the end of the reaction time, the chips were placed immediately into 25ml of sterile neutralizing solution containing 5.3g lecithin, 37.5g Tween 80, and 0.5g KH_2PO_4 in 1,000ml of distilled water.

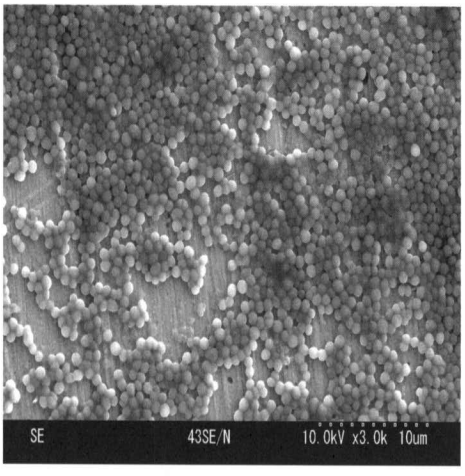
Control chips were placed directly into 25ml of neutralizing solution without exposure to detergent or



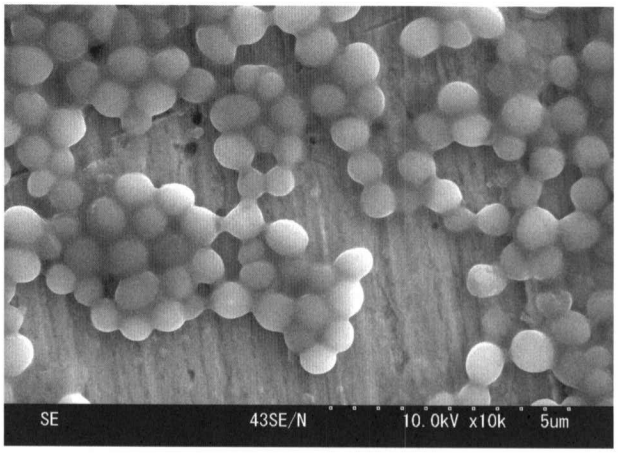
① Untreated stainless steel chip



② Chip treated with media alone



③ Biofilm *S. aureus* on chip ×3000



④ Biofilm *S. aureus* on chip ×10000

FIG. 1. Scanning electron microscopic findings of Biofilm *S. aureus* on stainless steel chip. (Accelerating voltage: 10.0kv)

sanitizer. Bacterial cells were removed from both sides of the neutralized chips by scraping them off with a Teflon scraper, followed by swabbing the scraper with a cotton-tipped swab, and rinsing the cotton swab with sterile PBS. The swab and rinsings were transferred to a sterile test tube and adjusted to a volume of 5.0ml with sterile PBS containing 0.1% (w/v) hexametaphosphate, and were stirred on a vortex mixer for 1 min. Viable cells in the PBS suspensions were enumerated by using spread plates of tryptic soy agar (TSA) with incubation at 37℃ for 48h. Each experiment was replicated twice.

The cleaning detergent formulations used in this study are usually diluted to the concentration of 0.075% according to the recommendation of manufacturers and are applied to the washing of the utensils and so on. Cleaning removes rather than inactivates microorganisms (Hood and Zottola, 1995). Usually, detergents have been used in food processing plants for cleaning various surfaces and instruments and the elimination of biofilm. When control experiments were carried out by submerging biofilm bacteria into 40ml of PBS (pH7.0) for 5 min, it was found that biofilm bacteria were not removed from the chips. Table 1 shows the removal of biofilm bacteria submerged in the 3 types of detergent solutions. It was found that 0.08% solution of any detergent was not effective at all against biofilm *E. coli, S.* Enteritidis, and *S. aureus*, when biofilm bacteria were submerged in the detergent solution. It was suggested that the removal effects of the detergents became stronger by the increase to the unpractical concentration of 1.5%, but the weakly alkaline detergent could not remove *E. coli* and *S.* Enteritidis even at higher concentrations. Thus, it was found that the biofilm bacteria could not be removed effectively from stainless steel chips only by submersion in any detergent at the commonly used concentrations.

Sodium hypochlorite is used widely as a strong oxidizing sanitizer in the food industry, and it is known to be very active in killing most bacteria, fungi, and viruses. Schwach and Zottola (1984) found that attached fibrils were not adversely affected by hypochlorite treatment and suggested that at least a detergent wash and a water rinse were needed before a hypochlorite treatment of the stainless steel surface to which bacterial cells were attached. Table 2 shows the inactivation of biofilm bacteria when exposed to hypochlorite solutions of active chlorine of 25 - 200 μg/ml for 5 min. As the active chlorine concentration became higher, more biofilm bacteria were inactivated, but none of the bacteria were completely inactivated. Even in a solution with an active chlorine of 200 μg/ml which is the maximal concentration usually used for the disinfection of the such surfaces as utensils and instruments, only 5.5, 3.9, and 2.0 log cfu/chip reduction was noted for *E. coli*, *S.* Enteritidis, and *S. aureus*, respectively.

Also, benzalkonium chloride, alkyldiaminoethylglycine hydrochloride, chlorhexidine digluconate, and polyhexamethylenebiguanide formulations are used for sanitizing the surfaces of utensils and instruments in the food industry at concentrations of 0.05 - 0.2%, 0.2 - 0.5%, 0.01 - 0.02%, and 0.1 - 0.2% as the active ingredient, respectively. Table 3 shows the inactivation of biofilm bacteria submerged in the 4 kinds of sanitizer solutions. Consequently, benzalkonium chloride solution inactivated *E. coli*, *S.* Enteritidis and *S.*

TABLE 1. Removal of biofilm bacteria by different detergents[1)]

detergent	conc. (%) of formulation	Bacterial counts (log of CFU) on biofilm:		
		E. coli O157	*S.* Enteritidis	*S. aureus*
Weakly alkaline detergent[2)]	0	7.3	4.9	6.8
	0.08	7.1	4.8	5.3
	0.15	7.4	4.7	5.3
	1.5	7.3	4.2	5.3
Neutral detergent[3)]	0	6.5	5.3	7.6
	0.08	6.3	5.4	7.6
	0.15	6.2	4.2	7.5
	1.5	5.5	4.3	6.5
Weakly acidic detergent[4)]	0	7.3	5.4	7.3
	0.08	6.9	5.2	7.3
	0.15	6.1	5.0	6.5
	1.5	5.9	3.7	6.3

1) Biofilm chips were submerged in the detergent solution for 5 min.
2) alkylether sulfate-alkylether amine oxide
3) alkyl glucoside
4) polyethylene alkylether-fatty acid alkanol amide-alkylether sulfate

aureus completely at the concentration of 0.1%. Alkyldiaminoethyl glycine hydrochloride at 0.5 to 4% could not inactivate biofilm bacteria completely. The 0.05% chlorhexidine digluconate solution inactivated completely *S.* Enteritidis only, but permitted a slight survival of *E. coli* and *S. aureus*. Regarding polyhexamethylene-biguanide, the 0.5% solution inactivated completely *E. coli* and almost *S.* Enteritidis, but there was noted only a ca. 1.8 log cfu reduction of the *S. aureus* population. Although the susceptibility of the planktonic cells to the sanitizers was considered to be not different among *S. aureus, E. coli*, and *S.* Enteritidis, the biofilm *S. aureus* population was shown to be more tolerant to each sanitizer than *E. coli* and/or *S.* Enteritidis at the same initial population levels.

Microorganisms that band together in biofilms are known to be protected from being killed by sanitizers and other antimicrobial agents (Frank and Koffi, 1990; Stewart et al., 2004). Generally the sanitizing agents are developed on the basis of tests using planktonic bacteria, which are quite different from the biofilm bacteria due to their altered physiological status (Wirtanen and Mattila-Sandholm, 1993). Because biofilm bacteria are resistant to chemical sanitizers, it is important to wash and sanitize thoroughly and routinely the surfaces of utensils and instruments for preventing the formation of biofilm.

TABLE 2. Inactivation of biofilm bacteria by sodium hypochlorite solution[1].

conc. of formulation	Bacterial counts (log of CFU) on biofilm:		
	E. coli O157	*S.* Enteritidis	*S. aureus*
0 μg/ml	6.9	5.2	5.2
25	6.2	4.3	4.0
50	4.2	3.6	3.7
100	2.6	2.7	3.4
200	1.4	1.3	3.2

1) Biofilm chips were submerged in the detergent solution for 5 min.

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TABLE 3. Sanitizing effects of different sanitizers against biofilm bacteria[1].

Formulation	conc. (%) of		Bacterial counts (log of CFU) on biofilm:		
	Formulation	Test solution	*E. coli* O157	*S.* Enteritidis	*S. aureus*
Benzalkonium chloride	10	0	5.2	5.2	5.3
		0.05	ND[2]	ND	1.0>[3]
		0.1	ND	ND	ND
		1.0	ND	ND	ND
Alkyldiaminoethyl glycine hydrochloride	40	0	5.8	6.5	6.5
		0.5	1.0>	1.0>	1.9
		1.0	1.0>	1.0>	1.5
		2.0	1.0>	1.0>	1.0
		4.0	1.0>	1.0>	1.0>
Chlorhexidine digluconate	20	0	5.2	5.2	5.1
		0.05	1.0>	ND	1.0>
		0.1	1.0>	ND	1.0>
		1.0	1.0>	ND	1.0>
Polyhexa-methylenebiguanide hydrochloride	20	0	5.1	6.1	6.2
		0.5	ND	1.0>	4.4
		1.0	ND	1.0>	1.9
		2.0	ND	1.0>	1.0>

1) Biofilm chips were submerged in the sanitizer solution for 5 min.
2) ND: not detected
3) 1.0> : less than 10 cfu

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