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Efficacy of Sanitizers on *Listeria*, *Salmonella*, and *Pseudomonas* Single and Mixed Biofilms in a Seafood Processing Environment

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ABSTRACT

Control and elimination of biofilm formation in the food processing environment is vital for food safety. This study was designed to investigate the efficacy of hydrogen peroxide (H_2O_2) pre-treatment combined with the regular daily cleaning procedure used in a shrimp plant to control biofilm formation. Single and mixed species biofilms of *Listeria*, *Salmonella* and *Pseudomonas* were used as the test model. Single biofilms on stainless steel (SS) coupons were formed under nutrient stress and harvested at 3 and 7 days to assess four cleaning procedures. Using 2% alkaline detergent for 10 minutes followed by two types of quaternary ammonium compounds (QACs) - based sanitizers completely eliminated single biofilms of *Listeria* and *Salmonella*. When alkaline was replaced to acidic type, microbial reduction achieved 5 log colony forming units (CFU)/cm^2 (or more). For the mixed species biofilm study, biofilms were formed under the simulated seafood processing plant conditions for 7 days, on SS, Teflon and rubber coupons. After pre-treated mixed species biofilms with H_2O_2 at 1% and 2% for 5 and 10 minutes followed by the regular cleaning procedure, 2% of H_2O_2 for 10 minutes reduced microorganisms by 6 log CFU/cm^2. Mixed biofilm on SS was easier to remove compared to the other surfaces. Overall these results suggest that the application of H_2O_2 prior to the regular cleaning process in food processing facilities may help to reduce and control biofilm formation, particularly biofilms composed of mixed species.

Keywords: Biofilm, *Listeria*, *Salmonella*, *Pseudomonas*, hydrogen peroxide, cleaning, sanitizer, stainless steel, Teflon, rubber

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INTRODUCTION

Microorganism contamination on food products has been increasing in the food processing environment even though routine cleaning and sanitizing using various detergents and chemicals is employed (Bridier *et al.*, 2015; Food & Water-Watch, 2007; Norhana *et al.*, 2010). Contaminated food causes harm to consumer's heath and economic issues as well as losses related to the brand name of the respective food producer (Bohme *et al.*, 2013). Biofilm formation is a serious concern due to inappropriate cleaning process (Brooks and Flint, 2008; Chmielewski and Frank, 2003; Giaouris *et al.*, 2014; Gibson *et al.*, 1999). Moreover, not only spoilage bacteria

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(e.g., *Pseudomonas*, *Klebsiella*) but also most of the foodborne pathogens (e.g., *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, *E. coli* H7: O157) are able to adhere to biofilms on most materials and under almost all of the environmental conditions in food production plants (Beauchamp *et al*., 2012; Bridier *et al*., 2015; Giaouris *et al*., 2012; Joseph *et al*., 2001; Marchand *et al*., 2012; Ryder *et al*., 2007). In addition, viable microorganisms in biofilm are tolerant to sanitizers because some microorganisms have specific mechanisms to resist sanitizing agents. A few examples include *Pseudomonas* spp., *Listeria* spp., *Salmonella* spp., *Escherichia coli*, etc. (Bridier *et al*., 2015; Chmielewski and Frank, 2003; Dourou *et al*., 2011; Duong, 2012; Ibusquiza *et al*, 2011; Myszka and Czaczyk, 2011). Furthermore, extracellular polymeric substances (EPS), which are produced by microorganisms to protect themselves against other species or under stress growth conditions, act as glue which help microorganisms to effectively adhere on the surfaces of equipment, leading to the failure of the removal of the biofilm during the cleaning process (Bridier *et al*., 2015; Giaouris *et al*., 2012; 2013). Therefore, finding the appropriate method for removing biofilm is the interest of both food scientists and food manufacturers. Numerous studies on evaluation of the effectiveness of sanitizer on single biofilms of both pathogenic and spoilage bacteria have been reported (Beauchamp *et al*., 2012; Belessi *et al*., 2011; Choi *et al*., 2012; Elmali *et al*., 2012; Ölmez and Temur, 2010; Oz *et al*., 2012). However, research on the efficacy of sanitizers to remove the mixed species biofilms at a mature stage remain limited, especially mixed species biofilm formation under simulated food processing ecosystem conditions. In addition, most research only focuses on the effect of sanitizers to eliminate organisms in biofilms (Aase *et al*., 2000; Fatemi and Frank, 1999; Joseph *et al*., 2001; Norwood and Gilmour, 2000). There is still a lack of studies that fully applies the simulation of likely cleaning process at the seafood processing facility, using both cleaning with detergent and disinfectant with sanitizers. Furthermore, employing a method to degrade the EPS in a biofilm before cleaning procedure has not been fully investigated (Giaouris *et al*., 2014; Xavier *et al*., 2005). Therefore, the objectives of this study were focused on the assessment of cleaning process at food processing plants through single mature biofilm formation under nutrient stress followed by a proposed modified cleaning method that combines EPS degradation by H_2O_2 and a cleaning process to assess the effectiveness of removing a mixed species biofilms under simulated food processing ecosystem conditions.

MATERIALS AND METHODS

Sanitizers and detergents

Sanitizers and detergents were supplied by a local seafood processing facility in Thailand where these chemicals are regularly used in cleaning process. These included an alkaline foam cleaner (Superp foam), acidic foam cleaner (Dilac Z – descaler), QACs (Spectrum- broad spectrum liquid disinfectant), PAA, oxidizing disinfectant- peracetic acid (Zal Perax II) and QACs- based (Quatdet clear -broad disinfectant, fogging). These chemicals were products of Diversey Hygiene (Thailand) Co., Ltd. Hydrogen peroxide (grade AR) was a product of QReC, New Zealand.

Bacteria cultures and stock preparation

Three types of bacteria (*Listeria monocytogenes* 101; *Salmonella enterica* serovar Aberdeen and *Pseudomonas aeruginosa*) were obtained from the Department of Food Science and Technology, Agro-Industry Faculty, Kasetsart University, Thailand. These bacteria (*Listeria monocytogenes* 101, *Salmonella enterica* serovar Aberdeen and *Pseudomonas aeruginosa*) were commonly present on foodborne pathogenic and spoilage bacteria, especially in seafood processing (Gram and Huss, 1996; Gram *et al*., 1987; Koonse *et al*., 2005; Norhana *et al*., 2010). All bacterial species were cultured (37°C, 24 h) and subcultured (37°C, 18 h) individually in 10 mL tryptic soy broth (TSB; Difco). Cells of the individual cultures were then harvested by centrifugation (10000 ×g at 4 °C for 10 min) followed by washing twice in 1 mL

phosphate buffered saline (PBS, pH 7.4) (Bae *et al.*, 2012; Stewart and Costerton, 2001). Washed cell pellets of each species were resuspended in 1 mL TSB. To prepare the single species stock, the resuspension of each species was mixed with sterilized glycerol 36% by a ratio of 1: 1 (v/v). Finally, stock cultures were stored in a refrigerator at -20°C.

Similar with the method to apply for preparing single species stock, the mixed species stock, which was used for the mixed species experiment, was prepared with 80% of volume cell suspension of *Pseudomonas* spp., 10% of volume of *Listeria* spp., and 10% of volume of *Salmonella* spp. Following this, the mixed species were added to glycerol and kept in a refrigerator at -20°C.

Biofilm formation

Single species biofilm formation

Stocks of three species *Listeria* spp., *Salmonella* spp., and *Pseudomonas* spp. were individually cultured (37°C, 24 h) and sub-cultured (37°C, 18 h) again to prepare single species biofilms for this experiment. Sterilized SS coupons (304, finish # 2B) with dimensions of 5 × 2 × 0.08 cm were transferred to 50 mL conical centrifuge tubes containing 40 mL of TSB (1% or 10%; Difco); which were inoculated with a 0.1mL suspension of sub-cultured bacterial cells; the bacterial population in each sample was approximately 2×10^7 CFU/mL. Following this, samples were incubated at 16°C for 3 days or 7 days. At sampling time (3 days or 7 days) single species biofilms were collected to conduct the experiments.

Mixed species biofilm formation under a simulated food processing ecosystem (SFP)

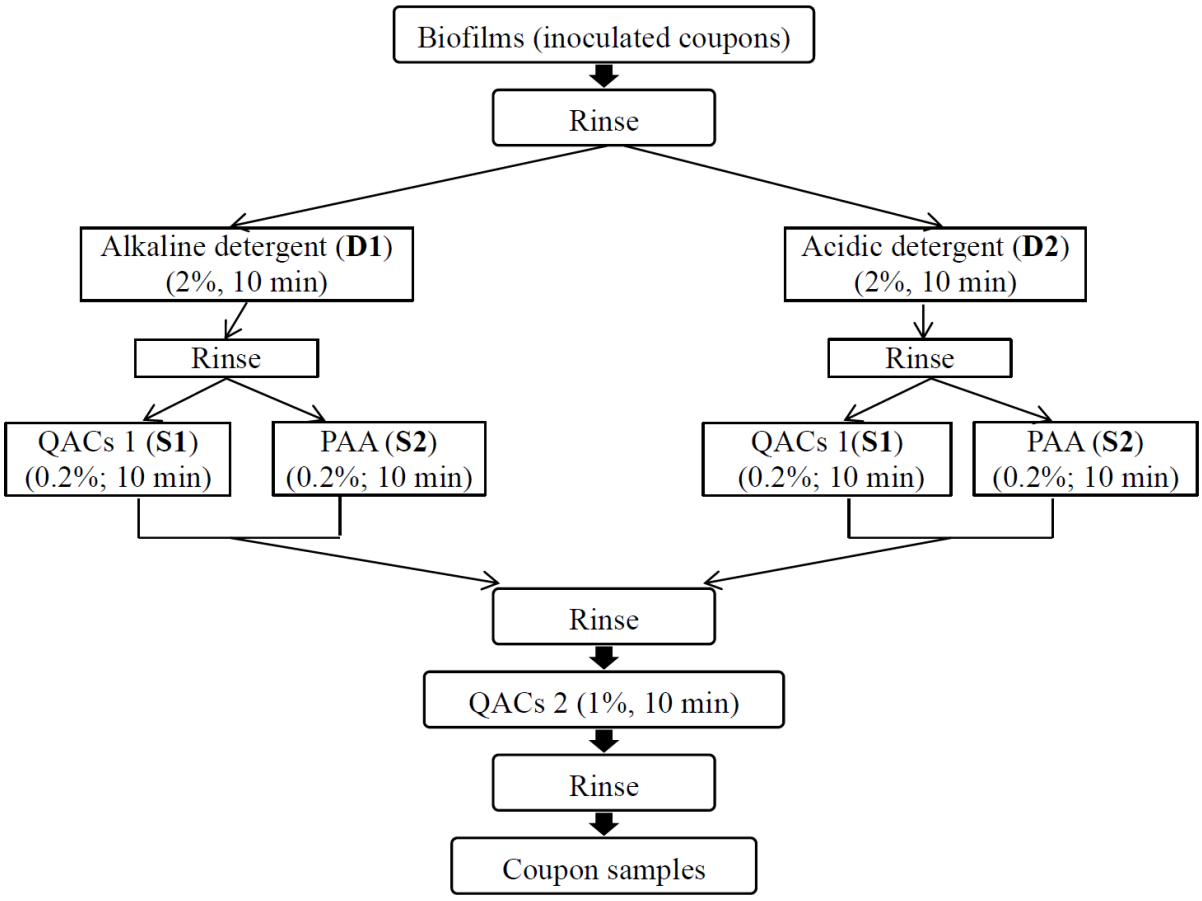
The SS coupons (304, finish # 2B); teflon® coupons and rubber coupons representing common materials that have been employed in food processing systems were used in this study. A modified CDC biofilm reactor (Centers for Disease Control, CDC; BioSurface Technologies Corp., Bozeman, MT, USA) (modified from Hadi *et al.*, 2010) was used for conducting this experiment. It included two main components, namely, a bioreactor and a frame holding coupons. Forty eight coupons were set up under the same conditions. First, coupons were soaked overnight in commercial detergent solution, degreased with 70% ethanol, thoroughly rinsed with tap water and distilled water (Hoa *et al.*, 2015), and coupons were subsequently vertically placed sequentially at coupon gaps on a frame of the biofilm reactor (bioreactor). These CDC bioreactors were autoclaved at 121° C for 15 min prior to use (Pan, 2005; Stewart and Costerton, 2001). Next, four liters of broth (1% TSB, 16°C) and 1 mL of mixed species stock were added into the bioreactor. The bacterial population in the bioreactor was approximately 4.4×10^6 CFU/mL. Following this, the bioreactor was placed in an incubator for 7 days, at 16°C under simulated food processing ecosystem with daily released and refreshed 4 liters of broth. Bacteria in the biofilm were subjected to broth for 8 hours/day, followed by starvation for nearly 16 hours /day. Finally, mixed species biofilms were harvested for conducting the experiment.

Cleaning process and modified cleaning process by pretreatment with hydrogen peroxide

Cleaning process

The cleaning process used at the seafood company was simulated for this experiment. The regime for the cleaning process consisted of rinsing, cleaning with detergent, rinsing, disinfecting, rinsing, re-disinfecting and rinsing. The four cleaning processes were respectively, (Fig. 1), D1S1- regular daily cleaning; D1S2- weekly cleaning; D2S1 and D2S2 - bi-monthly cleaning. To perform the cleaning process, biofilm coupons were placed in a cleaning holding coupon frame which had been designed for assessing cleaning performance. The cleaning procedure was conducted as indicated by the flow chart shown in Fig. 1. The spraying method was applied for all steps of each treatment. Distilled water, detergents, sanitizers and hydrogen peroxide were contained in the same type of bottles (distilled water bottle 16 oz # 500 mL). Spraying distance was approximately 10 cm from the top of bottle to coupons. Spraying time was approximately 30 seconds per treatment (4 cou-

Figure 1. Flow chart of four cleaning processes (D1S1) daily; (D1S2) weekly; (D2S1 & D2S2) bi-monthly



pons and both sides of coupons). Spraying volume was approximate 60 mL per treatment. At the end of each step, samples were covered and placed in a laminar flow biological safety cabinet for specified exposure times. The concentration of detergents, concentration of sanitizers and exposure time were determined based on the flow chart of each cleaning process. Detergents and sanitizers were diluted and placed in the refrigerator for an hour prior to the cleaning process. After the cleaning process, biofilm coupons were collected in duplicate and placed into sterilized petri dishes for evaluating the efficacy of each cleaning process.

Modified cleaning process by pre-treatment with H_2O_2

Similarly, a modified cleaning process was applied the pre-treatment with H_2O_2 before cleaning with a detergent-based stage. The pre-treatment with H_2O_2 was performed with two levels of concentrations (1% and 2%) and two levels of exposure time (5 and 10 minutes). Following this, biofilm coupon continuous performances were evaluated over a regular daily cleaning process (D1S1). For D1S1 samples (no pre-treatment with H_2O_2), coupons were rinsed with 10 mL distilled water, followed by continuous performance evaluations over a regular daily cleaning process (D1S1).

Determination of number of surviving bacteria

Surviving bacteria on inoculated coupons with single species or mixed species bacteria were detached carefully using two cotton swabs. For control samples (Biofilm, no cleaning process), coupons were rinsed twice with 10 mL distilled water in order to remove the loosely attached cells. Following this,

all samples were swabbed thoroughly and the swab heads were broken off into a glass tube containing 10mL sterilized saline peptone water (SPW) (0.85% of salt and 0.1% of bacteriological peptone, Difco; Bagge-Ravn *et al.*, 2003; Gibson *et al.*, 1999). Next, suspensions were left for 30 minutes at 16°C to recover cells after treatment with the sanitizing agent. The bacteria on the swabs were re-suspended by vortexing for 1 min at high speed (Vortex Genie 2 G – 560E, speed 8). The re-suspension fluid was serially diluted in SPW and spread in duplicate on Tryptic soy agar (TSA) (Difco). The TSA plates were incubated at 37°C for 1 day and bacterial viability was quantified (Bae *et al.*, 2012; Nguyen and Yuk, 2013).

Data analysis

Results of experiments were converted into log values and averages were reported. Mean values and standard deviations were determined for results of two biological independent tests in duplicates.

To calculate means and standard deviations, a value of 0.69 was assigned to sample outcomes that were below the lower limit of detection (<10 CFU/mL) of the spread method. This value was equal to 100 CFU/1 coupon (# 20 cm^2), or 5 CFU/cm^2. SPSS version 18 was used for statistical analyses. Statistical significance was set at P-value less than 0.05.

RESULTS AND DISCUSSION

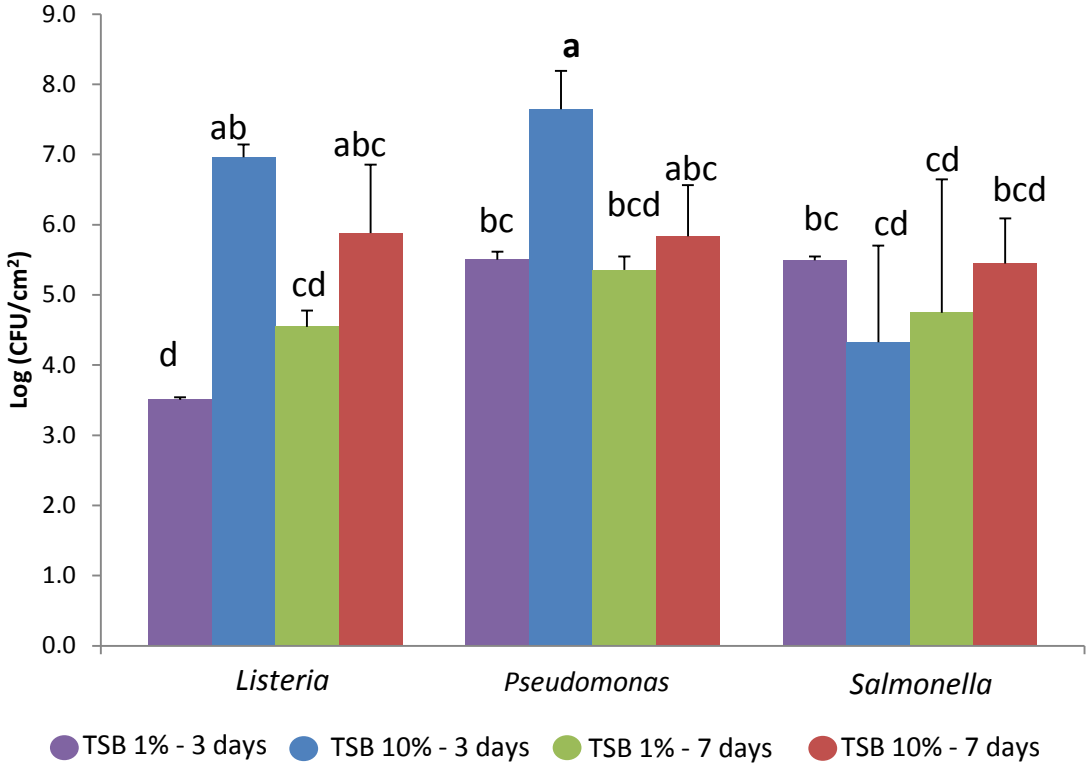
Assessment of efficacy of four cleaning processes by removing the differences of mature single species biofilms

Effect of growth conditions on biofilm population

Listeria spp., *Salmonella* spp., and *Pseudomonas* spp. have been demonstrated to be significant hazards in food production environments, especially in seafood processing ecosystems (Ababouch *et al.*, 2005; Bridier *et al.*, 2015; Giaouris *et al.*, 2014; Van Houdt and Michiels, 2010). In addition, *L. monocytogenes*, *Salmonella* spp. and *Pseudomonas* spp. have been widely studied and shown to have considerable ability to form single and mixed species biofilms (Bridier *et al.*, 2015; Dourou *et al.*, 2011; Giaouris *et al.*, 2013; Slama *et al.*, 2012). However, the ability to eliminate bacteria in biofilms depends on the characteristics of the biofilms and the cleaning process. In this experiment, biofilms which were formed on SS with two conditions of nutrient stress (TSB 10% or TSB 1%) and two different ages of biofilms (3 days or 7 days) and three species bacteria at 16 °C. These results are demonstrated in Fig. 2. Four cleaning procedures which have been used in the seafood plant were applied to examine the efficacy of each cleaning process.

Populations of *Listeria*, *Pseudomonas* and *Salmonella* biofilms are presented as log CFU/cm^2 values. Figure 2 presents the population of single species biofilms of *L. monocytogenes*, *Salmonella* spp., and *Pseudomonas* spp. ranging from 3.5 log CFU/cm^2 to 7.7 log CFU/cm^2. In general, it was observed that the population of cells in biofilm depended on the type of cultures and nutrient levels (TSB 10% and TSB 1%). There was no significant difference in the population of cells for 3 days biofilms or 7 days biofilms. Similar results were obtained from a study of Giaouris *et al.* (2013) for *Pseudomonas putida* at 18°C. Their study showed that during a 10 day sampling interval, *Pseudomonas* putida generated two biofilm-formed cycles, one reached on day 4 and another at day 8. The cell population was approximately 7 log CFU/cm^2. The results of this experiment indicated that the population of *Pseudomonas* cells was significantly higher than the population of *Salmonella* cells, but there was no significant difference with *Listeria* population in TSB 10%. This result may be explained due to the temperature of this experiment. In this experiment, the temperature of biofilm formation was set up at 16°C to simulate the working temperature of the seafood processing plant. Therefore, this temperature may not have influenced the growth of *Listeria* or *Pseudomonas* since they are psychrotrophic microorganisms, whereas *Salmonella* is a mesophilic bacteria. Moreover, due to the presence of a complex enzymatic system, *Pseudomonas* spp. can metabolize various materials around them to serve as their nutrient sources (Franzetti and Scar-

Figure 2. Population of viable cells in single biofilms of *Listeria*, *Pseudomonas* and *Salmonella* for 3 days or 7 days in TSB 1% and 10%, at 16°C. Results were expressed as mean values ± standard deviation from two independent tests in duplicate. Among different nutrient conditions, incubation times and type of cultures (a, b, c); the mean values with the same letters are not significantly different ($p>0.05$).



pellini, 2007). Hence, under nutrient stress in TSB 1%, the population of *Pseudomonas* biofilm reached approximately 5.5 log CFU/cm^2 whereas the *Listeria* biofilms reached only 3.5 and 4.5 log CFU/cm^2 for 3 days and 7 days biofilms, respectively.

Efficacy of four cleaning processes on different single biofilms

We applied cleaning procedures (Fig. 1), that were used in the seafood factory in daily, weekly and bimonthly. This procedure included: 1. rinsing with fresh water, 2. cleaning with 2% alkaline or acidic detergent for 10 min, 3. rinsing with fresh water to remove detergent, 4. sanitizing with QACs 1 or PAA 0.2% for 10 min, 5. repeating the rinsing process with fresh water to remove the sanitizers, 6. re-sanitizing by fogging with Quatdet clear - a QACs 2 based 1% for 10 min. Finally, equipment was rinsed with fresh water again and allowed to dry before production.

Among the four cleaning processes, treatment D1S1 was the least effective and treatment D2S2 was the most effectiveness for eliminating bacteria in biofilms (Table 1 and Table 2). In terms of efficacy to remove biofilm of various single species, the results show that the *Pseudomonas* biofilms had the highest ability to survive after the cleaning process followed by *Salmonella* and *Listeria*. All the cleaning processes were effective at removing biofilms of *Salmonella* and *Listeria*. According to Somers and Wong (2004) a combination of cleaning and sanitizing was more effective than sanitizer alone in terms of eliminating *Listeria monocytogenes* biofilms. Moreover, QACs, which was used in four cleaning processes as sanitizer and re-sanitizer, was more effective against *Salmonella* spp. (Gram–negative) (Sinde and Carballo, 2000) and *Listeria* spp. (Gram-positive) (Buffet-Bataillon *et al.*, 2012); even though they differ in their cell wall characterstics. Therefore the *Pseudomonas*

Table 1. Efficacy of 4 cleaning processes on different single biofilms of *Pseudomonas, Listeria, Salmonella* for 3 and 7 days in TSB 1% and TSB 10%

Organism	Time (days)	TSB (%)	log (CFU/cm^2)				
			Treatment				
			Biofilm	D1S1	D1S2	D2S1	D2S2
Pseudomonas	3	1	5.50 ± 0.11	2.63 ± 0.01^{ab}	0.79 ± 1.11^{ce}	ND	ND
Listeria	3	1	3.51 ± 0.04	0.70 ± 0.00^{c}	ND	0.70 ± 0.00^{c}	ND
Salmonella	3	1	5.49 ± 0.06	ND^{d}	ND	ND	ND
Pseudomonas	3	10	7.65 ± 0.54	4.13 ± 1.19^{a}	2.87± 0.66^{ab}	1.55 ± 1.20^{bc}	ND
Listeria	3	10	6.96 ± 0.38	0.70 ± 0.00^{c}	ND	0.70 ± 0.00^{c}	ND
Salmonella	3	10	4.33 ± 1.38	ND	ND	ND	ND
Pseudomonas	7	1	5.36 ± 0.19	1.74 ± 0.13^{bc}	0.59 ± 0.83^{ce}	0.35 ± 0.49^{ce}	0.35 ± 0.49^{ce}
Listeria	7	1	4.55 ± 0.23	ND	ND	ND	ND
Salmonella	7	1	4.75 ± 1.90	ND	ND	ND	ND
Pseudomonas	7	10	5.84 ± 0.73	2.71 ± 0.75^{ab}	0.50 ± 0.71^{ce}	1.61 ± 0.81^{bc}	0.70 ± 0.00^{c}
Listeria	7	10	5.88 ± 0.98	ND	ND	ND	ND
Salmonella	7	10	5.45 ± 0.64	ND	ND	ND	ND

[a,b,c] significant decrease within 4 cleaning processes (D1S1, D1S2, D2S1 and D2S2) in both rows and columns

[d] ND, not detectable, less than 0.69 log CFU/cm^2

[e] one sample detectable

Results were expressed as mean values ± standard deviation from two independent tests in duplicate. The mean values with the same letters are not significantly different ($p>0.05$).

biofilm would be considered a main concern. The results in Table 1 and Table 2 showed that treatment D1S1 and D1S2 removed only 3 to 4 log CFU/cm^2 *Pseudomonas*; whereas treatment D2S1 or D2S2 removed 5 to 6 log CFU/cm^2 *Pseudomonas*. The main difference between the four treatments was the types of detergents; treatment D1S1 and D1S2 using alkaline detergent, (D1- superp foam, 2%) and treatment D2S1 and D2S2 using acidic detergent (D2 - Dilac Z, 2%).

The characteristics of different bacteria, which contribute to the formation of their corresponding biofilms, affects their survival under the cleaning and disinfecting process was examined in the current study. Our results were similar to the results obtained by Gibson *et al*. (1999). The investigators showed that *Pseudomonas aeruginosa* was more resistant to the detergent products, greater than 3 log CFU/cm^2 still remained on SS coupons after cleaning; their research also reported that the maximum removal cells was a 4 log CFU/cm^2 reduction (Gibson *et al*., 1999). The reason *Pseudomonas* biofilms were more resistant than *Listeria* and *Salmonella* biofilms may be because of the difference in colonization mechanisms (Gibson *et al*., 1999). *Pseudomonas aeruginosa* attached to surfaces produces a variety of extracellular polymeric substance (EPS) such as cellulose, alginate, Pel and Psl exopolysac-

Table 2. Percentage reduction of 4 cleaning processes on different single biofilms of *Pseudomonas, Listeria, Salmonella* for 3 and 7 days in TSB 1% and TSB 10%

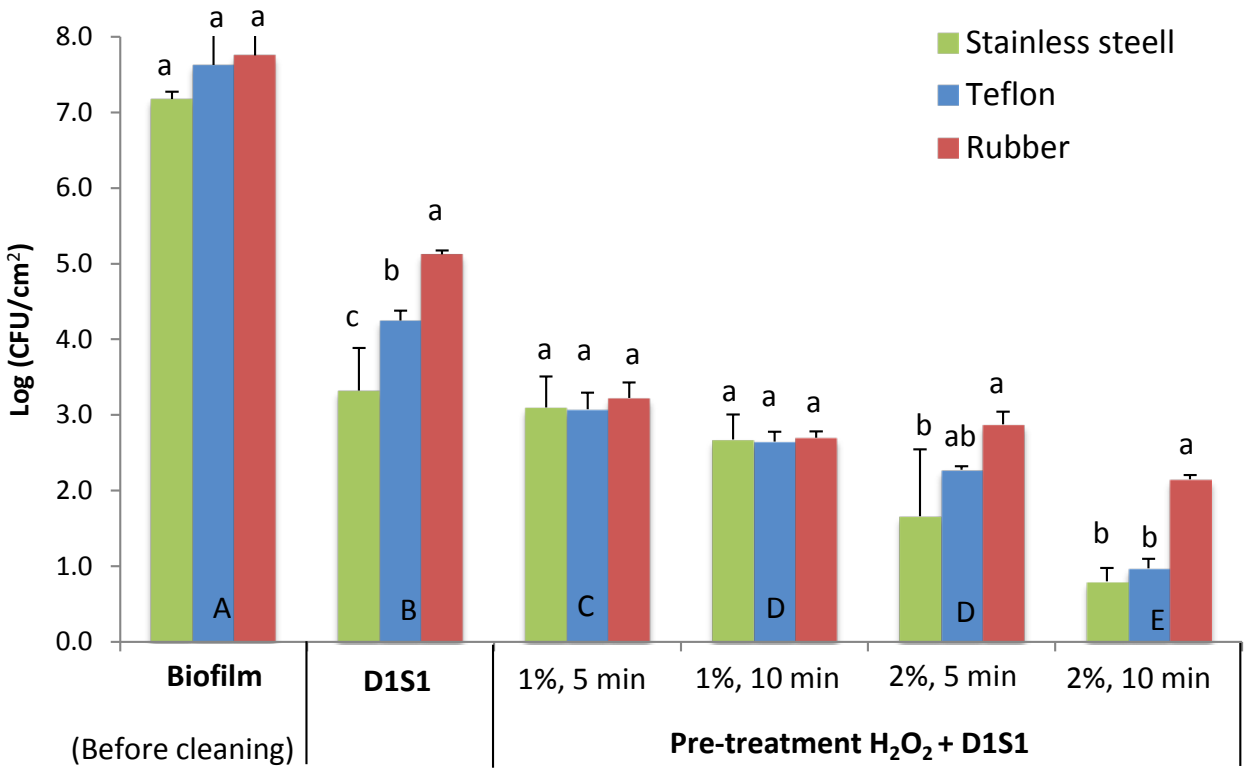
Organism	Time (days)	TSB (%)	Percentage reduction*			
			Treatment			
			D1S1	D1S2	D2S1	D2S2
Pseudomonas	3	1	99.8685	99.9943	100.0000	100.0000
Listeria	3	1	99.8435	100.0000	99.8435	100.0000
Salmonella	3	1	100.0000	100.0000	100.0000	100.0000
Pseudomonas	3	10	99.9235	99.9980	99.9998	100.0000
Listeria	3	10	99.9999	100.0000	99.9999	100.0000
Salmonella	3	10	100.0000	100.0000	100.0000	100.0000
Pseudomonas	7	1	99.9764	99.9968	99.9989	99.9989
Listeria	7	1	100.0000	100.0000	100.0000	100.0000
Salmonella	7	1	100.0000	100.0000	100.0000	100.0000
Pseudomonas	7	10	99.9232	99.9996	99.9935	99.9996
Listeria	7	10	100.0000	100.0000	100.0000	100.0000
Salmonella	7	10	100.0000	100.0000	100.0000	100.0000

*Each value is a mean of duplicate replication of two independent tests.

charides which help them to strongly adhere and form strong biofilms on surfaces (Giaouris *et al*., 2013; Gibson *et al*., 1999). Moreover, EPS also forms a matrix around the cells, which can protect the cells from adverse conditions (Gibson *et al*., 1999). In addition, the larger population of *Pseudomonas* meant thicker biofilm compared to those of *Listeria* or *Salmonella* biofilms, causing less diffusion of sanitizers in *Pseudomonas* biofilms. Therefore, *Pseudomonas* spp. may survive better than the others under the same treatment (Giaouris *et al*., 2013). The results revealed that under simultaneous growth conditions (3 days - TSB 1% or 7 days - TSB 10%) and having a similar cellular populations (approximately 5.5 log CFU/cm^2), there was significant differences in terms of viable cells of *Pseudomonas* and *Salmonella* biofilms; *Pseudomonas* spp. remained at approximately 2.7 log CFU/cm^2 while no *Salmonella* spp. colonies were recovered. Similar results were demonstrated by Giaouris *et al*. (2013). Gram-negative *P. putida* showed higher tolerance to benzalkonium chloride (BC- QACs -based) compared with the Gram positive *L. monocytogenes*.

When comparing the effectiveness of biofilm removal based on detergent pH, an acidic detergent (Dilac Z, pH 1.6; treatment D2) was more effective than an alkaline product (Superp foam, pH 12.2; treatment D1) in terms of effect on cell viability. Sim-

Figure 3. Efficacy of combination of H_2O_2 pre-treatment and regular daily cleaning process (D1S1) on mixed species biofilms on SS, Teflon and rubber. Results were expressed as mean values ± standard deviation from two independent tests in duplicate. Among different biofilm and cleaning processes (A, B, C, D, E) and among different materials at the same cleaning process (a, b, c); the mean values with the same letters are not significantly different (p>0.05).



ilarly, Gibson *et al.* (1999) found that the acidic detergent reduced the viable population of attached *Staphylococcus aureus* but not *P. aeruginosa*. Our results may be explained by source of *Pseudomonas*, *Pseudomonas* genus which can be found as an bundance species in fish and seafood ecosystem grew at pH 6 to 9; they did not growth at pH 4 (Shivaji *et al.*, 1989) thus leading to acidic detergent being more effective in cleaning than the alkaline detergent.

Indeed, PAA may have a stronger effect to antimicrobial activity compared with QACs, because PAA had both oxidizing and low pH functions in killing bacteria whereas QACs had only one antimicrobial mechanism, namely, interaction with cell membranes, disruption of membranes integrity and leakage of cellular content (Buffet-Bataillon *et al.*, 2012; Giaouris *et al.*, 2013).

Assessment cleaning and sanitizing procedure based on the efficacy to remove mixed species biofilms

Although combining detergent and sanitizer in the cleaning process showed more effectiveness to eliminate biofilms (Somers and Wong, 2004), *Pseudomonas* biofilms were still present on SS coupons after the cleaning process, particularly the D1S1 method. Hence, it can be concluded that the regular daily cleaning process D1S1 is an improper cleaning procedure to remove *Pseudomonas* biofilms. However, the cleaning process D1S1 was still applied daily because alkaline detergents have a higher potential to remove organic material and prevent the corosion of equipment in the food procesing environment. In addition, the ability to eliminate micro-

organisms in biofilms depends on many factors such as the microbial population of the biofilm, quorum sensing, and material where biofilms form and inhabit. From previous experiments, it was demonstrated that mixed species biofilms may be formed by numerous different species. Moreover, mixed species biofilms are usually more stable than single species biofilm (Giaouris *et al.*, 2014). Consequently, there was a need to evaluate the efficacy of cleaning process on mixed species biofilms of *Listeria*, *Salmonella* and *Pseudomonas* under simulated food processing conditions in the current experiment. According to the result from Gram *et al*. (1987), Guðbjörnsdóttir *et al*.(2005) and our previous experiment (data unpublished), the microflora in seafood plants was shown to be 80% Gram negative microorganisms; *Pseudomonas* spp. was shown to be the predominant species. Therefore, the mixed species stock which included 80% of volume cell suspension of *Pseudomonas* spp., 10% of volume of *Listeria* spp., and 10% of volume of *Salmonella* spp. was used for this experiment. The efficacy of the cleaning process was determined by the surviving bacteria attached on the surface of coupons after performing the cleaning process. The results were reported as a mean with duplicate replication of two independent experiments.

The modified cleaning process was applied to remove mixed species of 7 day biofilms on different substratum such as SS, Teflon and rubber. Biofilm formation on these materials ranged from 7.2 to 7.7 log CFU/cm^2. There were no significant difference in the population of cells adhering on SS, Teflon and rubber for 7 days biofilms. D1S1 sample consisted of no treatment with H_2O_2 using only the cleaning process of D1S1. Overall, the surviving bacterial cells of all samples decreased when H_2O_2 concentration and exposure time were increased (Fig. 3). Without pretreatment with H_2O_2, viable cells on SS, Teflon and rubber were 3.3, 4.3 and 5.2 log CFU/cm^2, respectively. The low efficacy for removing mixed species biofilms may be attributed to the large population of microbial cells in the biofilms, and interactions between cells and surfaces (Giaouris *et al.*, 2013, 2014; Lagha *et al.*, 2014; Norwood and Gilmour, 1999). Moreover, limiting the diffusion of sanitizers within the biofilm occurred because these strong biofilms formed under nutrient limitation (they were subjected to TSB 1%, 8 h per day) and 7 days.

Indeed, Giaouris *et al*. (2014) reported that one of the four mechanisms leading to increase resistance of organisms with sanitizers is a physical barrier formed by the EPS matrix. Therefore, disruption EPS matrix will increase the effectiveness of the cleaning procedure by increasing the penetration of sanitizer into biofilms (Simões *et al.*, 2010; Xavier *et al.*, 2005). There are several methods which could be used to destabilize the EPS matrix of biofilms including biological methods, physical methods and chemical methods; among them H_2O_2 (chemical method) was selected because of its advantages, namely, low-cost, ease of use, minimal impact on the environment, broad spectrum capabilities and high potential to degrade the EPS matrix (Back *et al.*, 2014; Gao *et al.*, 2014; Imamura *et al.*, 2010). According to Imamura *et al*. (2010) H_2O_2 molecules degenerate and produce °OH radicals by accepting an electron from the metal surface. Due to an extremely high oxidation potential, °OH radicals are generated on the metal surface at high concentrations (Imamura *et al.*, 2002). As a consequence, organic substances, which include in EPS, are instantaneously oxidized. The oxidized substances become soluble fragments which can easily be removed from surfaces by a regular cleaning procedure (Imamura *et al.*, 2002, 2010).

The effectiveness of pretreatment with H_2O_2 is associated with H_2O_2 concentration and exposure time. Average survival of microbial cells in different substratum ranged from 3.1 to 1.3 log CFU/cm^2 followed by H_2O_2 1% for 5 minutes and H_2O_2 2% for 10 minutes, respectively. The results showed that there was more than a 6 log CFU/cm^2 reduction in terms of pre-treatment with H_2O_2 2% for 10 minutes. A similar result was obtained by DeQueiroz and Day (2007). In their study, when sodium hypochlorite was combined with H_2O_2, cell numbers were reduced by 5 log to 6 log of *P. aeruginosa* biofilms after 1 min exposure while sodium hypochlorite reduced viable numbers by 3 log to 4 log under an equivalent concentration. In addition, *P. aeruginosa* biofilm

formation on SS and aluminum surfaces were also removed (DeQueiroz and Day, 2007). The results of this experiment was supported by Choi *et al*. (2012) where pathogen biofilms on the SS surfaces were reduced when treated with aerosolized hydrogen peroxide-based sanitizer. However, there was no significant difference between pre-treatment with H_2O_2 1% for 10 minutes and H_2O_2 2% for 5 minutes. It was suggested that the treatment of 1% of H_2O_2 for 10 minutes or 2% of H_2O_2 for 5 minutes should result in a low concentration or short exposure time for °OH radicals to decompose the EPS matrix of the highly mixed microbial species generated in these biofilms. Attachment or detachment of microorganisms on surfaces depends on both characteristics of bacteria and material surfaces (Sinde and Carballo, 2000). Among the three materials SS, Teflon and rubber, there were no significant difference in the number of adhesive cells but there was a significant decrease in the number of viable cells during the cleaning process. The number of viable cells on rubber remained high in both the control and the pre-treatment with H_2O_2. In fact, when applying the pre-treatment H_2O_2 2% for 10 minutes to the biofilm formation on the rubber coupon, there was greater than a 5 log CFU/cm^2 reduction, however there remained 2 log CFU/cm^2 of viable cells on the surfaces of rubber coupons. While applying the same treatment condition, biofilms on SS and Teflon were removed, reaching a 6 log CFU/cm^2 reduction. Higher efficacy in cleaning process to SS and Teflon could be explained because a high concentration of °OH radicals was produced on the metal surface (Imamura *et al*., 2010) and the more hydrophobic characteristics of the Teflon surface.

CONCLUSIONS

This study demonstrated the efficacy of combination of H_2O_2 pre-treatment with the regular daily cleaning procedure used in a shrimp plant to control biofilm formation. For a single biofilm, the population of bacteria in biofilms depended on bacteria characteristics and nutrient availability. *Pseudomonas* biofilms demonstrated higher adaptability for all growth conditions; as indicated by the higher number of cells in biofilms. Four existing cleaning processes were effective against *Listeria* and *Salmonella* biofilms, with *Pseudomonas* biofilms being the exception. For mixed-species biofilm, when combined with pretreatment of H_2O_2, the cleaning process was more effective when compared to using the existing cleaning process alone; there was a 4 log CFU/cm^2 reduction for the control method compared to a 6 log CFU/cm^2 reduction for the method that combined pre-treatment H_2O_2 2% for 10 minutes and a cleaning process for cleaning mixed species biofilms on SS coupons. SS is ideally suited for food industry, especially in terms of eliminating and preventing biofilm formation. Therefore, the combination of H_2O_2 pre-treatment and cleaning process can be used as an alternative method to remove mixed species biofilms in food processing equipment.

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