BIOFILM FORMATION UNDER VARIOUS TEMPERATURE CONDITIONS AND ITS REMOVAL TECHNIQUE

森松, 和也
九州大学大学院生物資源環境科学府環境農学専攻

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MORIMATSU KAZUYA

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BIOFILM FORMATION UNDER VARIOUS TEMPERATURE CONDITIONS AND ITS REMOVAL TECHNIQUE

By

MORIMATSU KAZUYA

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Postharvest Science Laboratory

Graduate School Bioresource and Bioenvironmental Sciences

Kyushu University

Japan

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AND ITS REMOVAL TECHNIQUE

ABSTRACT

Recently, there has been a high demand for food safety because of frequent food-poisoning incidents. In order to provide customers with safe food products, it is necessary to control microorganisms present on a product. However, the attached bacterial cells forming biofilm would come to have excessive tolerance against sterilization. Thus, the presence of bacteria forming biofilm has received much attention as a problem related to food safety. The objectives of this study were to investigate the characteristics of bacterial biofilm formation under various temperature conditions and to explore optimal concentrations and pH values of sodium hypochlorite (NaOCl) against bacteria forming biofilm. The thesis consists of introduction, three main chapters and conclusion.

In chapter 1, the background of the thesis was presented.

In chapter 2, the effect of temperature fluctuation on a bacterial biofilm formation was investigated in a single culture of Salmonella enterica, Staphylococcus aureus and Pseudomonas putida. In the single culture, biofilm formation of Sal. enterica and Sta.
Sta. aureus was enhanced even at a low temperature through temperature fluctuation while that of *Pseudomonas putida* at a low temperature was inhibited by temperature fluctuation. Moreover, in the result of investigation in a mixed culture of each of *Sal. enteica* and *Sta. aureus* with *P. putida*, a bacterial interaction between *P. putida* and each of *Sal. enterica* and *Sta. aureus* was induced by a stress of poor nutrient at high temperature, resulting in an acceleration of biofilm formation. This acceleration of biofilm formation was not affected considerably by temperature fluctuation, while it seemed that temperature fluctuation can enhance the attachment of *Sal. enterica* and *Sta. aureus*.

In chapter 3, the investigation focused on the effect of temperature on biofilm formation was conducted. In the result, it was shown that a maximum amount of attached biofilm was higher at low temperature than at high temperature in rich nutrient condition. In addition, mature biofilms after it reached the maximum amount were detached at high temperature while the mature biofilms remained on the attachment surface at low temperature. However, these effects of temperature on bacterial biofilm formation could be minimized by the lack of nutrients.

In chapter 4, the purpose of this research was to explore the optimum concentrations and pH values of NaOCl treatment against bacteria forming biofilm. An increase in the available chlorine concentrations and the pH values of NaOCl solution accelerated a
removal of the attached biofilm, especially, the treatment at a strong alkaline state of NaOCl solution gave the quickest removal rate of the biofilm. However, in regardless of conditions of NaOCl treatment, the attached biofilm partly remained attachment after NaOCl treatment. Moreover, the success and failure of sterilization with NaOCl treatment were vague.

In chapter 5, conclusion of this study was proposed.
AKNOWLEDGEMENT

First and foremost, I would like to express my sincere thanks to Prof. Toshitaka Uchino, my supervisor for his valuable guidance and support throughout the course of this research work. Indeed, without him, this study would not have been possible. I also extend my grateful thanks to Prof. Eiji Inoue, Laboratory of Bioproduction Engineering and Associate Prof. Fumihiko Tanaka, Laboratory of Postharvest science at Kyushu University, Assistant Prof. Daisuke Hamanaka for their intimate reviews and constructive suggestions for the draft of this dissertation.

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CHAPTER 1

Introduction

1.1. BACKGROUND OF RESEARCH

Recently, there has been a high demand for food safety because of frequent food-poisoning incidents. In order to provide customers with safe food products, it is necessary to control microorganisms present on a product surface. Therefore, a lot of researches on food safety, for example, sterilizing microbe and predicting microbial growth, has been conducted actively (Bratchelle et al., 1990, Wijtzes et al., 2001, Kim et al., 2003, Christensen et al., 2008). Although most of researches target at planktonic cells, many microbial cells attach onto a material surface in a real environment of a food production process, and the attached cells sometimes form biofilm (Jessen et al., 2003) (Fig. 1.1). In the process of biofilm formation, extracellular polysaccharide is produced to embed the attached cells, which would come to have excessive tolerance against
Bacterial attachment (1).
Bacterial growth and biofilm formation (2).
Biofilm detachment (3).

Fig.1.1 Process of biofilm formation
several stresses such as heat and chemical treatment (Zottola et al., 1994, Chmielewski et al., 2003). The microbial cells in biofilm show a different growth rate from planktonic cells, as pointed out by Kinniment et al. (1992). From these facts, a treatment of the previous research utilizing the planktonic cells sometimes can not control the bacteria forming biofilm. Thus, the presence of bacteria forming biofilm has received much attention as a problem related to food safety, and the researches on the bacteria forming biofilm were promoted in order to control the problems caused by bacterial biofilm. It was reported that bacterial attachment and biofilm formation in food processing factory can be inhibited by modifying a characteristics of the equipment surface (Wang et al., 2003, Nishioka et al., 2004). However, this method cannot be applied to the food surface treatment. In addition to these suggestions, Costerton et al. (2001) suggested several treatments against bacteria forming biofilm, however, these treatments have not been developed sufficiently for ensuring food safety. Houdt et al. (2010) also promoted the need for better understanding of the bacterial mechanism of biofilm
formation in order to control the problems caused by bacterial biofilm formation. Therefore, it is important for ensuring a high safety of food to gain more understanding of the characteristics of biofilm formation under environment condition related with a production process in food industry and to explore the treatment against bacteria forming biofilm.

1.2. AIM OF THIS RESEARCH

This research was undertaken to investigate the effect of environment condition related with the process of food industry on bacterial biofilm formation and to explore the treatment against bacteria forming biofilm. Although unsteady condition is frequently observed during process of food industry, there have been no information on an effect of unsteady condition on bacterial biofilm formation. The study in chapter 2 investigated biofilm formation under fluctuating temperature condition which is frequently observed during food distribution. In the result, although a risk of
temperature fluctuation was recognized by the result of present study, an effect of temperature on bacterial biofilm formation was unclear (Morimatsu et al., 2009, 2010, 2012). In chapter 3, the investigation was focused on the effect of temperature on biofilm formation. As a result, bacteria formed a different amount of biofilm with culturing condition. Thus, in chapter 4, to explore the treatment sterilizing any bacteria forming biofilm regardless of amount of attached biofilm, bacteria within a different amount of attached biofilm was treated with hypochlorite sodium (NaOCl), which is popularly utilized for food sanitation. In chapter 5, the characteristics of biofilm formation under various temperature conditions were summarized and the treatment which control bacteria forming biofilm sufficiently was concluded.
CHAPTER 2

Effect of temperature fluctuation on biofilm formation

2.1. INTRODUCTION

Most of previous studies related with biofilm formation have focused on a single microbial strain and have been conducted under steady environmental culture conditions (Dewanti et al., 1995, Rinaudi et al., 2006, Rode et al., 2007, Lianou et al., 2012). However, there are many kinds of microbial strains on food in an actual situation, and any microbial biofilms are generally developed under multi-existent condition of various strains of microorganisms (Bagge et al., 2001). The thickness and stability of biofilms are sometimes enhanced by an interaction among various strains of microorganisms, as James et al. (1995) pointed out. An interaction between different bacterial species has been researched actively, and it was reported that the interactions can be classified as cooperation, competition, neutralism, and more (Haack et al., 1982, Sturman et al., 1994, Siebel et
Although most of these studies on the bacterial interaction have been also conducted under steady environmental culture conditions, the surroundings around the microorganisms frequently fluctuate. For example, a temperature fluctuation is observed during distribution of food and agricultural produce (Jacxsens et al., 2002, Uchino et al., 2006). However, there have been not many studies of biofilm conducted on these unsteady condition. Therefore, taking these environment in biofilm formation on the surface of food into consideration, the effect of the unsteady temperature condition on biofilm formation under a bacterial interaction in a mixed culture should be investigated.

The present study aims firstly to clarify the effect of temperature fluctuation on biofilm formation of Salmonella enterica, Staphylococcus aureus and Pseudomonas putida by examining biofilm formation in a single culture of these strains under conditions of constant temperature and fluctuating temperature, and secondly to study the effect of temperature fluctuation on the bacterial interaction by observing biofilm formation in the mixed cultures of P.
putida with each of Sal. enterica and Sta. aureus.

2.2. MATERIALS AND METHODS

2.2.1. Bacterial strains and growth conditions

The bacterial strains were Staphylococcus aureus. subsp. aureus NBRC 100910-derived strain from NITE Biological Resource Center (NBRC), Salmonella enterica. subsp. enterica NBRC 13245-derived strain from NBRC and Pseudomonas putida which was isolated from cucumber fruits and identified by analyzing base sequence of 16S-rDNA region using PCR method. Sta. aureus and Sal. enterica are one of the main food poisoning bacteria, P. putida is one of the genus Pseudomonas with a good ability to form biofilm. All strains were cultured in Tryptic Soy Broth (TSB) at 25 °C with agitation of 105 rpm for 72 hours.

2.2.2. Biofilm formation on an inner surface of polystyrene tube in a single culture

Biofilm formation on an inner surface of polystyrene tube
in a single culture was prepared according to the method proposed by Planchon et al. (2006) with some modifications. Each staphylococcal, salmonella and pseudomonal subcultures were separately diluted with sterile distilled water until optical density (OD) at 600 nm was adjusted to a value of 1.0 by a spectrophotometer (V-530, JASCO Corporation) so that initial bacterial count in the single culture of *Sta. aureus*, *Sal. enterica* and *P. putida* were $5.8 \times 10^8$, $2.2 \times 10^8$ and $5.0 \times 10^8$ CFU/ml, respectively. An adjusted culture of 0.1 ml was distributed in a prepared 15 ml polystyrene tube with 5 ml of TSB at 100% or 5% diluted with sterile distilled water, in order to identify the effect of the nutrient concentration on biofilm formation. For bacterial attachment onto the inner surface of the tube, the samples were allowed to stand for 30 minutes at room temperature. The tubes were then incubated in an incubator for 5 days at constant and fluctuating temperature conditions in order for the microorganisms to form biofilm. The constant temperature conditions were set at 5 °C and 30 °C, which reflected a refrigerated condition during the food
distribution and a high temperature in summer. In addition, two patterns of fluctuating temperature condition were applied. For the pattern (i) of fluctuating temperature condition, the tubes were incubated at ca. 5 °C for 1 day, and after that these were incubated at ca. 30 °C for 4 days. For the pattern (ii), the tubes were incubated at ca. 5 °C for 1 day then at ca. 30 °C for 1 day, after that the tubes were incubated at ca. 5 °C for 3 days. (Fig. 2.1).

2.2.3. Biofilm formation on an inner surface of polystyrene tube in a mixed culture

Each staphylococcal, salmonella and pseudomonal subcultures were diluted with sterile distilled water in the same way as shown in 2.2.2.. Each staphylococcal culture and salmonella culture were mixed with pseudomonal culture so that initial bacterial count in the mixed culture of Sta. aureus and Sal. enterica with P. putida were $3.1 \times 10^8$ and $7.4 \times 10^8$ CFU/ml, respectively. Subsequent operation for biofilm formation on the inner surface of the polystyrene tube in the mixed culture was performed according to the method
Fig. 2.1 Profile of fluctuating temperature condition.
2.2.4. Quantification assay for an amount of attached biofilm

The quantification assay for an amount of attached biofilm was performed according to the method proposed by Stepanovic et al. (2000) with some modifications (Fig. 2.2 (a)). After the incubation, solution in each tube was drained. Then, sterile distilled water of 5 ml was distributed in each tube and drained to wash. Viable and dead cells with exopolysaccharides remaining onto the polystyrene tube after washing were defined as biofilm. Subsequently, the attached cells and exopolysaccharides on each tube were stained with 5 ml of 0.1% crystal violet solution for 10 minutes. After draining the solution, the inner surface was rinsed off by distributing and draining 5 ml of sterile distilled water and then dried in a clean bench. The bound dye was resolubilized with 5 ml of 99.5% ethanol by ultrasonication at 125 W - 42 kHz for 1 minute at room temperature. The OD of the solution obtained with the manner described above was measured at 500 nm using a spectrophotometer according to the method
(a) Quantification assay for an amount of attached biofilm

Washing with sterile distilled water → Staining with 0.1% crystal violet solution → Distributing 99.5% ethanol → Resolubilizing by ultrasonication → Measuring absorbance at 500nm

(b) Bacterial count in biofilm matrix of a mixed culture

Washing with sterile distilled water → Distributing sterile distilled water → Resolubilizing by ultrasonication → Mixing bacterial suspension with agar broth solution → Counting bacterial colonies after incubation

Fig.2.2 Outline of experimental method for quantification assay for an amount of attached biofilm and bacterial count in biofilm matrix of a mixed culture
described by Hamanaka et al. (2007). The quantification was performed in triplicate.

2.2.4. Bacterial count in biofilm matrix of a mixed culture

To investigate the change in microbial flora of the biofilm, the viable bacterial number in the biofilm matrix was counted as follows (Fig. 2.2 (b)). After the incubation, the solution in each tube was drained and washed once with 5 ml of sterile distilled water and dried in the clean bench. Subsequently, 5 ml of sterile distilled water was dispensed into each tube, and then the bacteria attached to the inner surface of the tube were resolubilized by ultrasonication at 125 W - 42 kHz for 1 minute at room temperature. After diluting the bacterial suspension, standard method agar broth for counting both of *P. putida* and *Sal. enterica* or *Sta. aureus*, X-SAL agar for *Sal. enterica* and mannitol salt agar for *Sta. aureus* was mixed with the bacterial suspension in a petri dish. Each of standard method agar, X-SAL agar and mannitol salt agar petri dishes were incubated at 25 °C for 48 hours, 37 °C for 24 hours and 30 °C for 48 hours, respectively. After the
incubation, the colonies on the agar were counted for calculating total bacterial count, staphylococcal count and salmonella count, respectively. The experiment was performed at least three times.

2.2.6. Statistical analysis

The mean value of OD and viable count for each day were statistically evaluated using the modified t-test based on Ryan's multiple test. ($P<0.05$).

2.3. RESULTS AND DISCUSSION

2.3.1. Biofilm formation in a single culture

In the single culture of Sal. enterica and Sta. aureus at low constant temperature of 5 °C (Fig. 2.3 (a), (b)), an amount of attached biofilm remained almost constant in all culturing days regardless of TSB concentration. Whereas, at high constant temperature of 30 °C (Fig.2.4 (a), (b)), the biofilm amount in both of the single cultures increased on the 1st day of culturing. Since a optimal temperature for growth of Sta.
Fig. 2.3 Amount of attached biofilm indicated by absorbance in the single cultures of *Sal. enterica*, *Sta. aureus*, and *P. putida* at 5 °C.
Fig. 2.4  Amount of attached biofilm indicated by absorbance in the single cultures of *Sal. enterica*, *Sta. aureus*, and *P. putida* at 30 °C.
aureus and Sal. enterica is 35 °C and 37 °C, a low temperature could prevent Sta. aureus and Sal. enterica from not only bacterial growth but also biofilm formation while Sta. aureus and Sal. enterica at a high temperature could form biofilm. However, a different effect on biofilm formation at fluctuating temperature condition of the pattern (i) was observed after a rise in temperature from 5 °C to 30 °C (Fig. 2.5 (a) (b)), which a significant acceleration of biofilm formation in comparison with high constant temperature was observed with 100% TSB in the single culture of Sal. enterica in contrast to Sta. aureus (P<0.05). Therefore, temperature fluctuation of the pattern (i) could accelerate biofilm formation of Sal. enterica at high temperature under rich nutrient condition. In the single culture of Sal. enterica from the 3rd day to the 5th day of culturing, a biofilm detachment as indicated by a decrease in the biofilm amount was observed at the pattern (i) in regardless of TSB concentration (Fig. 2.5 (a)), while the biofilm amount at the pattern (ii) remained almost constant in 100% TSB and rather increased in 5% diluted TSB (Fig. 2.6
Fig. 2.5 Amount of attached biofilm indicated by absorbance in the single cultures of *Sal. enterica*, *Sta. aureus*, and *P. putida* at fluctuating temperature condition of the pattern (i).
(a)). In addition, also in the single culture of *Sta. aureus* regardless of TSB concentration (Fig. 2.6 (b)), biofilm formation was shown after a declining from 30 °C to 5 °C at the pattern (ii) despite no biofilm formation at 5 °C of constant temperature condition. Thus, temperature fluctuation of the pattern (ii) could accelerate the biofilm formation of *Sal. enterica* and *Sta. aureus* even at low temperature. Meanwhile, in the single culture of *P. putida* in contrast to the other single cultures with both of TSB concentrations, an active biofilm formation was observed at low constant temperature while no considerable biofilm formation was observed at high constant temperature in all culturing days (Fig. 2.3 (c), Fig. 2.4 (c)). And, comparing between biofilm formation at low constant temperature and that at fluctuating temperature condition of the pattern (ii) (Fig. 2.3 (c), Fig. 2.6 (c)), it was seemed that temperature fluctuation of the pattern (ii) could prevent biofilm formation of *P. putida*. 
Fig. 2.6 Amount of attached biofilm indicated by absorbance in the single cultures of *Sal. enterica*, *Sta. aureus*, and *P. putida* at fluctuating temperature condition of the pattern (ii).

•: 100% TSB □: 5% diluted TSB
2.3.2. Biofilm formation in a mixed culture

In both of the mixed cultures, an increase in the amount of biofilm with cultivation times was observed at low constant temperature with both of 100% and 5% diluted TSB to be similar to that in the single culture of *P. putida* (Fig. 2.7, Fig. 2.8). In addition, regardless of TSB concentration, total bacterial count increased with cultivation time in both of the mixed cultures while staphylococcal count remained almost constant and salmonella count slightly decreased in each of the mixed culture. Therefore, in both of the mixed cultures, *P. putida* principally formed biofilm at 5 °C while *Sta. aureus* and *Sal. enterica* could neither form biofilm nor grow well in each of the mixed cultures, respectively. A bacterial interaction could not be recognized in both of the mixed cultures at the low constant temperature because the biofilm amount in both of the mixed cultures did not markedly increase in comparison with the single culture of *P. putida*.

At the high constant temperature in the mixed culture of *Sal. enterica*, the amount of attached biofilm in 100% TSB decreased from the 2nd day to the 5th day after a increase in
**Fig. 2.7** Biofilm amount indicated by absorbance and viable bacterial count in biofilm in the mixed culture of *P. putida* with *Sal. enterica* at 5 °C.

<table>
<thead>
<tr>
<th>Culturing time (day)</th>
<th>Biofilm amount (OD$_{500\text{nm}}$)</th>
<th>Viable bacterial count (log CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a) 100% TSB</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0.06</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0.12</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>0.18</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>0.24</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>0.24</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>(b) 5% diluted TSB</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0.06</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0.12</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>0.18</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>0.24</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>0.24</td>
<td>5</td>
</tr>
</tbody>
</table>
Fig. 2.8 Biofilm amount indicated by absorbance and viable bacterial count in biofilm in the mixed culture of *P. putida* with *Sta. aureus* at 5 °C.
the biofilm amount on the 1st day while a maintenance of the biofilm amount was observed in 5% diluted TSB after a increase in the biofilm amount until the 3rd day (Fig. 2.9). In the mixed culture of *Sta. aureus*, the biofilm amount in 100% TSB kept almost constant over the culturing period after a increase in the biofilm amount on the 1st day while a increase in the biofilm amount was observed from the beginning to the end of culturing in 5% diluted TSB (Fig. 2.10). In both of the mixed cultures, although a delicate different biofilm formation from all of the single cultures was observed under high concentration TSB condition, a significant difference between the biofilm amount in each of the mixed cultures and that in all of the single cultures was not observed. While in 5% diluted TSB, a significant increase in the amount of attached biofilm in comparison with each of the single cultures was observed in both of the mixed culture ($P<0.05$). Therefore, bacteria forming biofilm could survive under rich nutrient condition even without a bacterial interaction related with biofilm formation, while a stress of poor nutrient condition could induce the bacterial interaction and
Fig. 2.9 Biofilm amount indicated by absorbance and viable bacterial count in biofilm in the mixed culture of *P. putida* with *Sal. enterica* at 30 °C.
Fig. 2.10 Biofilm amount indicated by absorbance and viable bacterial count in biofilm in the mixed culture of *P. putida* with *Sta. aureus* at 30 °C.
accelerate bacterial biofilm formation. In the mixed culture of *Sal. enterica* using 5% diluted TSB, an acceleration of biofilm formation from the beginning to the 3rd day of the culturing might be a bacterial interaction related with Quorum-sensing system, in which bacteria produce a signal material for recognizing a density of bacteria. Gram-negative bacteria, such as *Sal. enterica* and *P. putida*, produce N-acyl-homoserine lactones (AHLs) as a signal material to enhance biofilm formation, and AHLs works between different bacterial strains, even salmonella bacteria and pseudomonal bacteria (Kjelleberg *et al.*., 2002). Therefore, *Sal. enterica* and *P. putida* could cooperate through Quorum-sensing system to form biofilm, in the results, the same tendency of a change of total bacterial count and salmonella count was observed in all culturing days. Whereas, in the mixed culture of *Sta. aureus* with 5% diluted TSB throughout an acceleration of biofilm formation from the 3rd day to the 5th day, total bacterial count increased while staphylococcal count decreased. One of the studies for the interaction was reported by Qin *et al.* (2009) that *P.
*aeruginosa* exopolysaccharides disrupted the establishment of *Sta. epidermidis* biofilm in order to compete more successfully for nutrients. Thus, in this study, *P. putida* might also form exopolysaccharides to disrupt staphylococcal biofilms and decrease staphylococcal count in order to compete more successfully with *Sta. aureus* for nutrients.

At fluctuating temperature condition of the pattern (i) in the mixed culture of *Sal. enterica* under low TSB concentration (Fig. 2.11), an increase in the biofilm amount similar to the high constant temperature was observed from the 1st day to the 3rd day after the temperature rise. In addition, also in the mixed culture of *Sta. aureus* under low TSB concentration (Fig. 2.12), an acceleration of biofilm formation induced by the competitive interaction was observed. However, a significant difference between biofilm formation at high constant temperature and the pattern (i) was not observed. Therefore, the temperature rise could have no effect on bacterial interaction related with biofilm formation. However, in the mixed culture with *Sta. aureus* in 100% TSB, an experimental result related with effect of temperature rise on
Fig. 2.11 Biofilm amount indicated by absorbance and viable bacterial count in biofilm in the mixed culture of *P. putida* with *Sal. enterica* at fluctuating temperature condition of the pattern (i).
Fig. 2.12 Biofilm amount indicated by absorbance and viable bacterial count in biofilm in the mixed culture of *P. putida* with *Sta. aureus* at fluctuating temperature condition of the pattern (i).
the structure of biofilm was obtained. A significant decrease in the biofilm amount occurred from the 2nd day to the 3rd day of cultivating after the temperature rise in contrast to constant temperature conditions ($P<0.05$), this decrease in the biofilm amount could be due to biofilm detachment. Although it was not clearly why biofilm detachment occurred after the temperature rise, one of the reasons behind biofilm detachment might be the acceleration of biofilm formation rate induced by increasing temperature. Banks et al. (1991) reported that the difference of bacterial biofilm formation rate in the dual species bacterial biofilm formation experiment affects the structure of biofilm. The faster-growing biofilm species dominates the upper layer of the biofilm, while the slower-growing species is relegated to the lower layers. In this study, at 5 °C on the 1st day, *P. putida* could be considered as the faster-growing biofilm species and occupied the upper layer. However, after the temperature rose to 30 °C on the 2nd day, the biofilm formation rate of *Sta. aureus* was accelerated. As a result, *Sta. aureus* at the lower layer might invoke and mix with *P. putida*
in the upper layer of *P. putida*. Then, the upper layer of *P. putida* may have been entangled with staphylococcal detachment because of considerable decrease in staphylococcal count from the 2nd day to 4th day. And, in the mixed culture of *Sal. enterica* for 100% TSB, a biofilm detachment occurred as exposed to high temperature for a shorter period at the pattern (i) than at high constant temperature, this detachment might also be caused by the mixing bacterial layer. However, this hypothesis about an effect of temperature rising on biofilm structure was not clearly demonstrated from the result of this study. Therefore, hereafter, it is necessary that this hypothesis, which stated that the temperature rise could affect a structure of biofilm and accelerate biofilm detachment, is demonstrated by actual observation of biofilm structure for example utilizing confocal laser microscopy.

Under the condition of fluctuating temperature of the pattern (ii), although both of the biofilm amounts in 100% and 5% diluted TSB increased from the 2nd day to the 5th day after temperature declined (Fig. 2.13), this increase in the
Fig. 2.13 Biofilm amount indicated by absorbance and viable bacterial count in biofilm in the mixed culture of *P. putida* with *Sal. enterica* at fluctuating temperature condition of the pattern (ii).
biofilm amount was less than that at the low constant temperature in the mixed culture of *Sal. enterica*. Thus, an effect of temperature fluctuation of the pattern (ii) could inhibit the biofilm formation at low temperature. And, in the mixed culture of *Sta. aureus*, although the competitive interaction accelerated biofilm formation as observed at high constant temperature and the pattern (i), a significant difference of the biofilm amount among temperature conditions was not recognized (Fig. 2.14) \( (P<0.05) \). Therefore, temperature fluctuation of the pattern (ii) had no promoting effect on biofilm formation in the both of the mixed cultures. However, focusing on salmonella count and staphylococcal count in each of the mixed cultures at fluctuating temperature condition of the pattern (ii), a significant difference between the low constant temperature and the pattern (ii) was observed. In the mixed culture of *Sal. enterica*, the salmonella count was significantly high in comparison with that at the low constant temperature conditions in all culturing days \( (P<0.05) \). And, in the mixed culture of *Sta. aureus*, a decrease in staphylococcal count
Fig. 2.14 Biofilm amount indicated by absorbance and viable bacterial count in biofilm in the mixed culture of \textit{P. putida} with \textit{Sta. aureus} at fluctuating temperature condition of the pattern (ii).
induced by the competitive interaction was small in comparison with the pattern (i). Considering the result in the single cultures of *Sta. aureus* that temperature fluctuation of the pattern (ii) accelerated staphylococcal biofilm formation, staphylococcal biofilm in the mixed culture seemed to increase. As a result of this biofilm formation, bacterial detachment of *S. aureus* was prevented as it is generally stated that bacteria form biofilm to enhance bacterial attachment and also prevent bacterial detachment (Manuel *et al.*, 2010), and the staphylococcal count at the pattern (ii) remained high in comparison with the other temperature condition. Therefore, temperature fluctuation of the pattern (ii) could not favour an acceleration of biofilm formation in both of the mixed culture but a attachment of *Sal. enterica* and *Sta. aureus* at low temperature.

### 2.4. CONCLUSIONS

In this investigation, the aim was to evaluate an effect of temperature fluctuation on biofilm formation in the single
culture and the mixed culture. In the single culture, *Sal. enterica* and *Sta. aureus* could enhance the ability of biofilm formation at low temperature by temperature fluctuation, while *P. putida* could be inhibited from biofilm formation by temperature fluctuation. In the mixed culture, a stress of poor nutrient condition could induce a bacterial interaction between *P. putida* and each of *Sal. enterica* and *Sta. aureus* at high temperature. Although the bacterial interaction could accelerate biofilm formation, this acceleration of biofilm formation induced by the bacterial interaction could not be affected considerably by temperature fluctuation. However, it seemed that the attachment of *Sal. enterica* and *Sta. aureus* could be favoured by temperature fluctuation. In conclusion, it was indicated that an unsuitable temperature fluctuations in food distribution may have no considerable effect on biofilm formation under multi-existent condition of various strains of microorganisms. However, the temperature fluctuation may pose a high risk of food poisoning. Thus, it is important to maintain a constant temperature during food distribution.
CHAPTER 3
Effect of temperature on biofilm formation

3.1. INTRODUCTION

In chapter 2, the risk of temperature fluctuation during food distribution for food safety was recognized by observing biofilm formation under fluctuating temperature condition. However, the specific effect of temperature on microbial biofilm formation could not be identified, especially in the single culture of \( P. \) \( putida \). To gain a better understanding of the risk of biofilm formation under temperature fluctuation, the effect of temperature on biofilm formation should be clarified.

This study aims to investigate the effect of temperature on biofilm formation by examining an amount of attached biofilm and the number of bacteria in biofilm matrix at a variety of constant temperatures in the single culture of \( P. \) \( putida \).
3.2. MATERIALS AND METHODS

3.2.1. Bacterial strain and growth condition

In this chapter, *Pseudomonas putida* isolated from cucumber fruits in 2.2.1. was used to investigate the effect of temperature on biofilm formation, and this strain was cultured in TSB at 25 °C with agitation of 105 rpm for 48 hours.

3.2.2. Biofilm formation on a coupon of polyvinyl chloride

Biofilm formation on a coupon of polyvinyl chloride (10 × 10 mm, thickness = 1 mm) was performed according to the method proposed by Planchon *et al.* (2006) with some modifications. Pseudomonal subcultures were diluted with sterile distilled water until OD at 600 nm was adjusted to a value of 1.0 so that initial bacterial count was $6.0 \times 10^8$ CFU/ml. Adjusted culture of 0.05 ml was distributed in a prepared 15 ml polystyrene tubes with the polyvinyl chloride coupon and 2.5 ml of TSB of which concentration was 100% or 5% diluted with sterile distilled water. For bacterial
attachment onto the coupon, the samples were immersed in the prepared solution for 30 minutes at room temperature. The tubes were then incubated for 10 days at a constant temperature in order that microorganisms form biofilm. To investigate the effect of temperature on biofilm formation onto an attachment surface, the incubation temperatures were set at 5 °C, 10 °C, 20 °C and 30 °C, which reflect the temperature range encountered during actual food distribution.

3.2.3. Quantification assay for an amount of attached biofilm

The quantification assay for an amount of attached biofilm was performed according to the method proposed by Stepanovic et al. (2000) with some modifications. After the incubation, solution in each tube was drained. Then, sterile distilled water of 2.5 ml was distributed in each tube and drained to wash the coupon. Subsequently, the attached cells and exopolysaccharides on each coupon were stained with 2.5 ml of 0.1% crystal violet solution for 10 minutes. After draining the solution, the coupon surface was rinsed off by
distributing and draining 2.5 ml of sterile distilled water and then dried in a clean bench. The bound dye was resolubilized with 2.5 ml of 99.5% ethanol by ultrasonication at 125 W - 42 kHz for 1 minute at room temperature. The OD of the solution obtained with the manner described above was measured at 500 nm using the spectrophotometer. The quantification was performed in triplicate.

3.2.4. Bacterial count in biofilm matrix

After the incubation, solution in each tube was drained. Then, sterile distilled water of 2.5 ml was distributed in each tube and drained to wash the coupon. Subsequently, the bacteria attached onto the coupon were resolubilized with 2.5 ml of sterile distilled water by the ultrasonication for 1 minute at room temperature. After diluting the bacterial suspension, standard method agar broth was mixed with the bacterial suspension and the mixture was incubated at 25 °C for 48 hours to count the numbers of bacteria in biofilm matrix. The count was repeated at least three times.

3.2.5. Statistical Analysis

The mean OD value and viable count for each day and each temperature
condition were statistically evaluated using the modified t-test based on Ryan's multiple test ($P<0.05$).

### 3.3. RESULTS AND DISCUSSION

With 100% TSB in all temperature conditions, the amount of biofilm increased according to cultivation time until the biofilm amount reached a peak (Fig. 3.1). However, subsequently, a tendency of biofilm amount differed at each temperature condition. At 30 °C, after reaching a peak on the culturing 1st day, a decrease in biofilm amount was observed from the 1st day to the 1.5th day, then, biofilm amount became constant. The difference in OD between the 1st day and the 1.5th day was significant ($P<0.05$). At 20 °C, biofilm amount once increased by 1.5th day and became almost constant by 8th day, finally decreased. The difference in OD between 8th day and 10th day was significant ($P<0.05$). Whereas, at a low temperature of 5 °C and 10 °C, biofilm amount remained at a constant level after reaching a peak on the 5th day. Bacterial count in biofilm also increased to a
Fig. 3.1 Amounts of biofilm as indicated by absorbance of *P. putida* cultures incubated at different constant temperatures in 100% TSB.
peak and then remained constant in all temperature conditions with the exception of 30 °C (Fig. 3.2). At 30 °C, in contrast to the other temperature conditions, bacterial count in biofilm significantly decreased from the 2nd day to the 3rd day after reaching a peak ($P<0.05$), and then maintained constant. From these results, it was clarified that biofilm detachment as shown at high temperature was not observed after reaching a peak of biofilm amount at low temperatures, and the maximum biofilm amount decreased with increasing temperature. Taking into consideration that the viscosity of exopolysaccharides decreases as temperature increases (Lewis et al., 1989), biofilm detachment may be a result of the effect of temperature on the viscosity of exopolysaccharides. In addition, since the rate of bacterial growth increased with the temperature from the beginning of culture to the 1st day, pseudomonal activity indicated as the bacterial growth rate had a temperature dependency. Therefore, at a high temperature, the biofilm of *P. putida* can be led to detach through a decrease in the viscosity of exopolysaccharides and an increase in pseudomonal activity.
Fig. 3.2 Viable bacterial counts as a measure of *P. putida* biofilm formation in cultures incubated at different constant temperatures in 100% TSB.

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Fig. 3.2 Viable bacterial counts as a measure of *P. putida* biofilm formation in cultures incubated at different constant temperatures in 100% TSB.
Conversely, at a low temperature, that is a severe temperature condition for *P. putida*, bacteria remain attached on the attachment surface to better tolerate environmental stress, as reported by Poulsen *et al.* (1999).

With 5% diluted TSB, the biofilm amount was lower than with 100% TSB in all culturing days (Fig. 3.3). On the other hand, in chapter 2, a considerable biofilm amount of *P. putida* cultured in poor nutrient condition has been shown similar to that in rich nutrient. *P. putida* in chapter 2 was used immediately after isolated from cucumber fruits, while that in chapter 3 was used after long period of the subculture. Thus, *P. putida* in chapter 3 may lose its resistance to poor nutrient conditions because of long storage period for the subculture. In the case of bacterial biofilm formation under the effect of nutrient condition, bacteria form a thick biofilm in rich nutrient conditions. Conversely, very thin biofilm was formed under poor nutrient conditions (Wimpenny *et al.*, 1997). For this reason, a lack of nutrients can prevent *P. putida* from forming biofilm. Although a temperature dependency of pseudomonal activity indicated as the rate of
Fig. 3.3 Amounts of biofilm as indicated by absorbance of *P. putida* cultures incubated at different constant temperatures in 5% diluted TSB.

- ◆: 5 °C
- □: 10 °C
- ▲: 20 °C
- ○: 30 °C
bacterial growth was also observed similar to that of 100% TSB, no significant difference among all temperature condition with 5% diluted TSB was detected in the maximum amount of biofilm in contrast to 100% TSB (Fig. 3.4). Thus, a stress of poor nutrient condition not only prevented biofilm formation of *P. putida*, but also minimized the effect of temperature on biofilm formation. In addition, similar to the result obtained from using 100% TSB at 30 °C, a significant decrease in biofilm amount considered as the biofilm detachment was found at 5% diluted TSB for all temperature condition (*P*<0.05). Although a decrease in temperature can prevent biofilm detachment in 100% TSB as mentioned above, biofilm detachment also occurred at a low temperature in 5% diluted TSB. Thus, a lack of nutrients may enhance biofilm detachment even at low temperature conditions at which the viscosity of the exopolysaccharides is increased. The development of biofilm, including biofilm detachment, is regulated by cell-to-cell signals, known as the quorum-sensing system (Davies *et al.*, 1998). Therefore, the quorum-sensing system regulating biofilm detachment may be
Viable bacterial counts as a measure of *P. putida* biofilm formation in cultures incubated at different constant temperatures in 5% diluted TSB.

Fig. 3.4 Viable bacterial counts as a measure of *P. putida* biofilm formation in cultures incubated at different constant temperatures in 5% diluted TSB.
closely related to the nutritional condition. Thus, when there is a lack of nutrients, some biofilms undergo detachment to spread to new surfaces in search of nutrients, while other biofilms remain attachment to tolerate the stress associated with poor nutrient conditions.

3.4. CONCLUSION

The aim of this investigation was to identify an effect of temperature on bacterial biofilm formation. The results of present study showed that the maximum amount of attached biofilm was higher at low temperature conditions than at high temperature conditions in rich nutrient condition. In addition, the biofilm detached at high temperature after it reached the maximum amount while attachment of the biofilm remained unchanged at low temperature. On the other hand, under poor nutrient condition, a difference of the maximum biofilm amount among temperature conditions was not observed, moreover, biofilm detachment occurred at all temperatures. Therefore, the effect of temperature on bacterial biofilm
formation was minimized by the lack of nutrients.

From the result of this investigation, it could be clarified that a long period of low temperature storage induced considerably attached amount of biofilm in the case of a rich nutrient condition as a blot attaching onto the food surface. And, these situations in addition to an emergence of temperature fluctuation condition could lead to a risk that a quickly biofilm detachment was caused by exposure to high temperature, possibly resulting in a serious food poisoning accident. However, taking that a stress of poor nutrient condition minimized the biofilm formation into consideration, it was concluded that removal operation on a blot on the food surface might decrease a risk of biofilm detachment caused by temperature fluctuation.
CHAPTER 4

Effect of sodium hypochlorite treatment on biofilm forming bacteria

4.1. INTRODUCTION

It is well known that biofilm forming bacteria have a difficulty to be sterilized by bactericidal agents. In fact, some studies on the disinfection of biofilm forming bacteria have been conducted intensively, and many of these studies on disinfection against biofilm forming bacteria have aimed only to decrease the number of bacteria (Mitchell et al., 2008, Checinska et al., 2011, Park et al., 2012). Biofilm reacts with a bactericidal agent before the agent contacts with bacteria. As a result, biofilm forming bacteria have a difficulty to be disinfected because of a decrease in the concentration of the agent by the reaction with biofilm components (Samrakandi, 1997). From this idea, the amount of biofilm may affect disinfection against biofilm forming bacteria. Previous study has shown that the amount of attached biofilm differed by
culturing condition (Morimatsu et al., 2012), therefore, the
effect of disinfectant against biofilm forming bacteria may
also differ with culturing condition. Therefore, the effect of
different culturing condition of the attached biofilm on the
disinfection of biofilm forming bacteria should be
investigated. A residual biofilm might be observed after
disinfection even if disinfection against the biofilm forming
bacteria is sufficiently performed. As pointed by Simões et al.
(2006), there is a risk that surviving bacteria after
disinfection grow with utilizing the remaining biofilm which
consists of organic substances and exopolysaccharides as a
nutrient resource. For this reason, not only a disinfection
effect but also a removal effect of chemical agent on biofilm
forming bacteria and an attached biofilm should be
considered to explore the efficient method of controlling
biofilm forming bacteria. In this research, sodium
hypochlorite (NaOCl) as a disinfectant was focused, because
it has a wide bactericidal spectrum regardless of bacterial
species and a high capability for washing. However, the
disinfecting and washing capabilities of NaOCl depend on pH
values and the availability of free chlorine concentrations of NaOCl (Fukuzaki, 2005). The optimal concentrations and pH values of NaOCl should be explored to disinfect biofilm forming bacteria and wash out biofilm from bacterial attachment surface successfully.

This study aims to explore the optimal concentrations and pH values of NaOCl solution by investigating the disinfection of biofilm forming bacteria and the removal of biofilm by NaOCl treatment with various concentrations and pH values.

4.2. MATERIALS AND METHODS

4.2.1. Bacterial strain and growth condition

In this chapter, Pseudomonas putida isolated from cucumber fruits as described in 2.2.1. was used to investigate the effect of NaOCl treatment on biofilm forming bacteria, and this strain was cultured in TSB at 25 °C with agitation of 105 rpm for 48 hours.
4.2.2. Biofilm formation on a coupon of polyvinyl chloride

The experiment of biofilm formation on the coupon of polyvinyl chloride was performed according to the method described in 3.2.2. To compare the results of NaOCl treatment for a different amount of attached biofilm, culturing temperature, TSB concentrations and cultivating time were employed in the same manner as the conditions in chapter 3 shown in Table 4.1 so that the amount of attached biofilm becomes maximum or stabilized.

4.2.3. Conditioning sodium hypochlorite

An available free chlorine concentration of NaOCl was determined by DPD (N, N-diethyl-r-phenylenediamine) colorimetric method. Each of NaOCl was diluted with sterile distilled water so that an available free chlorine concentration was adjusted as 50ppm, 100ppm and 200ppm of NaOCl and corresponding pH values of these solutions were 9.8, 10.1 and 10.4, respectively. A value of pH was measured by a pH meter (HM-25R, TOA DKK). Hydrochloric acid or sodium hydrate was added to NaOCl of 50ppm in order to
Table 4.1 Cultivating time for each conditions

<table>
<thead>
<tr>
<th>Incubating temperature</th>
<th>Cultivating time (Days)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum amount</td>
<td>Stabilized amount</td>
</tr>
<tr>
<td></td>
<td>100% TSB</td>
<td>5% diluted TSB</td>
</tr>
<tr>
<td>5 °C</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>10 °C</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>20 °C</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>30 °C</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
adjust to pH values of 5.7 or 11.7, respectively.

4.2.4. Sodium hypochlorite treatment

After incubation, the bacterial solution in each tube was drained, and each tube was dispensed with 2.5 ml of sterile distilled water, draining again for washing the polyvinyl chloride coupon. The polyvinyl chloride coupon was immersed in 2.5 ml of each condition of NaOCl solution for 1, 3, 5 and 10 minutes. After that, 2.5 ml of sterile distilled water was added to the tubes and the liquids were drained for washing the polyvinyl chloride coupon.

4.2.5. Quantification assay for an amount of attached biofilm after sodium hypochlorite treatment

The amount of attached biofilm on the polyvinyl chloride coupon after NaOCl treatment was quantified by the method as described in 3.2.3. Quantification was performed in triplicate.
4.2.6. *Bacterial count in biofilm after sodium hypochlorite treatment*

The number of viable bacteria in the biofilm on the polyvinyl chloride coupon after NaOCl treatment were counted according to the method as described in 3.2.4.. The count was repeated at least three times.

**4.3. RESULTS AND DISCUSSION**

Fig. 4.1, Fig. 4.2 and Fig. 4.3 shows effect of NaOCl treatment time on the residual biofilm amount after the treatment of 50 ppm of available chlorine concentrations at the pH value of 11.7, 9.8 and 5.7, respectively. Since the amount of biofilm remained after NaOCl treatment for 1 minute decreased in comparison with the initial amount of the attached biofilm at all pH values of NaOCl solution, the treatment could remove a part of the attached biofilm immediately regardless of the pH values. A decrease in the residual biofilm amount according to extending treatment time was not observed at pH 5.7 of NaOCl solution. The treatment for 1 minute at pH 9.8 decreased the residual
Fig. 4.1 Effect of NaOCl treatment time on the residual biofilm amount after the treatment of 50 ppm of available chlorine concentrations at the pH value of 11.7.
Fig. 4.2 Effect of NaOCl treatment time on the residual biofilm amount after the treatment of 50 ppm of available chlorine concentrations at the pH value of 9.8.
Fig. 4.3 Effect of NaOCl treatment time on the residual biofilm amount after the treatment of 50 ppm of available chlorine concentrations at the pH value of 5.7.
biofilm amount to be similar to that after the treatment for 1 minute at pH 5.7. However, it was clear that increasing the treatment time prompted a removal of the biofilm and decreased the residual biofilm amount at pH 9.8, since the residual biofilm amount after NaOCl treatment for 10 minutes decreased in comparison with that after the treatment for 1 minute. At pH 11.7 of NaOCl solution, NaOCl treatment for 3 minutes decreased the residual amount in comparison with the treatment for 1 minute, but, no considerable removal of biofilm caused by the increase in the treatment time was observed. The treatment for 3 minutes at pH 11.7 decreased the residual biofilm amount to be similar to that after the treatment for 10 minutes at pH 9.8. The concentration of OH\(^-\) ion in washing solution has a great roll for removing fouling (Takahashi et al., 2006). Hence, an increase in the pH values increased the washing power of NaOCl solution. Therefore, the treatment at high pH values of NaOCl solution could remove biofilm in a shorter time than that at low pH value.

Fig. 4.4 and Fig. 4.5 shows effect of NaOCl treatment time on the residual biofilm amount after the treatment of 100 ppm
Fig 4.4 Effect of NaOCl treatment time on the residual biofilm amount after the treatment of 100 ppm of available chlorine concentrations at the pH value of 10.1.
Residual amount of biofilm after NaOCl treatment (OD\(_{500\text{ nm}}\))

Fig. 4.5 Effect of NaOCl treatment time on the residual biofilm amount after the treatment of 200 ppm of available chlorine concentrations at the pH value of 10.4.
and 200 ppm of available chlorine concentrations at the pH value of 10.1 and 10.4. In regardless of the free chlorine concentrations of 50 ppm, 100 ppm and 200 ppm, the residual biofilm amount decreased according to extending the treatment time. Comparing the residual biofilm amount after the treatment for 5 minutes among 50 ppm, 100 ppm and 200 ppm of the free chlorine concentrations, the amount decreased with an increase in the concentrations. A major factor for an efficient removal of fouling with NaOCl treatment is not only the pH value but also the concentrations of available chlorine (Urano et al., 2005). Therefore, an increase in the available chlorine concentrations of NaOCl solution could remove biofilm more quickly.

However, no considerable decrease in the residual biofilm amount after NaOCl treatment for 10 minutes was observed regardless of an increase in the concentrations of NaOCl solution at ca. pH 10. Moreover, also at pH 11.7 of NaOCl solution, although the residual biofilm amount after the treatment for 3 minutes was similar to be the amount after the treatment in all of the chlorine concentrations for 10 minutes,
a considerable decrease in the residual biofilm amount was not observed with an extension of the treatment time over 3 minutes. From these results, if the treatment time is extended more than 10 minutes, the residual biofilm could not be removed completely by NaOCl treatment regardless of the pH values and the chlorine concentrations. In regardless of NaOCl treatment condition, the residual amount of biofilm firstly increased with initial biofilm amount and then reached a constant value of about 0.05. When NaOCl solution was treated to remove the attached biofilm, NaOCl acted the surface layer of biofilm and washed the layer out. Then, the thickness decreased but NaOCl could not remove the biofilm directly attaches to coupon surface. Considering a process of biofilm attachment on the coupon, biofilm firstly could attach to a portion of coupon and gradually could extend over the whole surface flatwise as shown in Fig.4.6. After that, biofilm thickness could increase with an increase in the initial amount of biofilm while biofilm attachment area on the coupon could not increase with the increase in the initial biofilm amount. Accordingly, residual biofilm amount
Fig. 4.6 Diagram of the residual biofilm after NaOCl treatment under a different amount of the attached biofilm.
attached to whole surface of coupon constantly might remain after NaOCl treatment regardless of the treatment condition.

Fig. 4.7 shows viable bateria count in the residual biofilm amount after NaOCl treatment for 1 minute. After NaOCl treatment for 1 minute, in regardless of initial amount of attached biofilm, no detection of viable bacteria in biofilm was observed, and ca. 4 log CFU/ml of bacterial count was rarely observed. Taking into consideration that an initial bacterial count before NaOCl treatment was around 6 log CFU/ml, some viable bacteria in biofilm was sterilized completely by NaOCl treatment, whereas the other viable bacteria in biofilm randomly survived after NaOCl treatment. Active bacteria are distributed in the upper layer of biofilm while dormant bacteria are distributed in the bottom layer of biofilm because of decreasing in the concentrations of nutrient and oxygen according to a depth of biofilm layer. (Kim et al., 2009). Therefore, no detection of viable bacteria could be induced by removal of not only upper biofilm layer but also viable bacteria after NaOCl treatment. Whereas, a rare detection of viable bacteria after NaOCl treatment
Fig. 4.7 Viable bacterial count in the residual biofilm after NaOCl treatment for 1 minute.
might be induced by the existence of dormant bacteria in bottom biofilm layer which rarely resumes activity to grow after the removal of upper biofilm layer. However, it was unclear why the dormant bacteria randomly resumed activity to grow after the removal of all of viable bacteria with NaOCl treatment. Thus, this mechanism of resuming activity of dormant bacteria should be shed on light by further research.

4.4. CONCLUSIONS

The aim of this investigation was to explore the optimal concentrations and pH values of NaOCl solution as disinfectant against biofilm forming bacteria. In present study, the removal rate of the attached biofilm with NaOCl treatment could increase according to an increase in the available free chlorine concentrations and the pH values, especially, the treatment at a strong alkaline state removed the biofilm quickly. However, since a constant amount of residual biofilm after NaOCl treatment was observed regardless of condition and time of NaOCl treatment, the
biofilm which attached directly on the surface of the coupon could not be removed completely with NaOCl treatment. Moreover, the success and failure of sterilization with NaOCl treatment against the biofilm forming bacteria was vague in the present study. From these results, the further research should be conducted to explore the condition of NaOCl treatment which can lead to removing more amount of the attached biofilm and to sterilizing the biofilm forming bacteria completely.
CHAPTER 5

Summary and Conclusions

The studies presented in this thesis were performed to investigate the effect of temperature fluctuation on biofilm formation, to clarify the specific effect of temperature on biofilm formation and to explore the optimal concentrations and pH values of NaOCl solution as bactericidal agent against biofilm forming bacteria. The summary and conclusions for each chapter were addressed below.

In chapter 2, the effect of temperature fluctuation on biofilm formation was investigated in the single cultures and the mixed cultures. The results in the single cultures showed that biofilm formation of *Salmonella enterica* and *Staphylococcus aureus* were accelerated even at a low temperature through temperature fluctuation while that of *Pseudomonas putida* at a low temperature was inhibited by temperature fluctuation. In the mixed cultures, the stress of poor nutrient condition induced a bacterial interaction between *P. putida* and each of *Sal. enterica* and *Sta. aureus* at
high temperature. Although the temperature fluctuation affected the biofilm formation in the single cultures, there were no considerable effect of the temperature fluctuation on the biofilm formation in the mixed cultures. In contrast to effect of temperature fluctuation on biofilm formation, it seemed that the attachment of *Sal. enterica* and *Sta. aureus* on the inner surface of polystyrene test tube was promoted by temperature fluctuation. From these results, it was suggested that an unsuitable temperature fluctuation in food distribution might increase attachment of food poisoning bacteria and lead consumers to food poisoning accidents.

Subsequently, a clarification of effect of temperature on biofilm formation was made in chapter 3. The result of investigation showed that the maximum amount of attached biofilm was higher at a low temperature than at a high temperature in rich nutrient. In addition, a matured biofilm was detached at a high temperature after it reached the maximum amount, while the matured biofilm remain attachment at a low temperature. On the other hand, the lack of nutrients minimized this effect of temperature on bacterial
biofilm formation. From these results, it was clarified that bacterial biofilm formation could be prevented by not only a strict maintenance of low storage temperature but also washing treatment for removing a blot on the food surface.

In chapter 4, the purpose of this research was to explore the optimum available chlorine concentrations and pH values of NaOCl solution against biofilm forming bacteria. An increase in the available chlorine concentrations and the pH values of NaOCl solution accelerated a removal of the attached biofilm, especially, the treatment at a strong alkaline state of NaOCl solution gave the quickest removal rate of the biofilm. However, in regardless of conditions of NaOCl treatment, the attached biofilm partly remained after NaOCl treatment. Moreover, the success and failure of sterilization with NaOCl treatment were vague. From these results, although the treatment at the alkaline state of NaOCl solution could have a capability to remove a constant amount of the biofilm and to disinfect biofilm forming bacteria, hereafter, the best condition of NaOCl treatment which can lead to removing more amount of the attached biofilm and to sterilizing the
biofilm forming bacteria completely should be explored.

It was concluded that the optimal treatment with NaOCl solution could decrease the biofilm and the biofilm forming bacteria on the surface of food including fresh agricultural product, moreover, a strictly low temperature management during food distribution after the disinfection could prevent food-poisoning incidents caused by biofilm forming bacteria.
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