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Thesis for the Degree of Master of Engineering

Preparation of *Crassostrea gigas* Samples Bioaccumulated by *Vibrio vulnificus* and Its Depuration Using UV-light

by

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Pukyong National University

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(생물농축기법으로 비브리오 패혈증균을 인공감염시킨 굴 시료와 자외선을 이용한 오염패류 인공정화)

Advisor: Prof.Young-Mog Kim

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정희진

부경대학교대학원 식품공학과

요약

육상 오염에 취약한 연안에서 양식하는 굴은 여과섭이를 하는 특성으로 병원성 미생물 등의 오염물질이 중장선에 농축되고 생식으로 섭취하는 비중이 높아 오염된 굴의 섭취에 의한 식중독 사고가 빈번히 발생한다. 특히, 해수 유래의 병원성 비브리오균 감염과 관련된 발병률이 높은 것으로 보고되고 있다. 이에 병원성 미생물에 오염된 패류를 정화하기 위한 자연정화(relaying)와 인공정화(depuration)등에 대한 연구가 진행되고 있다. 본 연구에서는 해산물을 생식으로 섭취하는 비중이 높은 국내에서 특히, 치사율이 높은 *Vibrio vulnificus* (비브리오 패혈증균)에 의한 감염사고를 예방하기 위하여 인공정화에 의한 *V. vulnificus*의 저감화에 대하여 연구하였다. 이를 위하여 먼저, *V. vulnificus*를

생물농축기법(bioaccumulation)으로 생굴에 인위감염시킨 패류시료를 준비하고 인공정화에 의한 오염굴시료에서의 *V. vulnificus* 저감화 가능성에 대하여 분석하였다. 또한, 인공정화 중의 굴의 식품학적 품질변화에 대하여 모니터링 하기 위하여 생굴의 신선도와 관련된 품질평가로써 pH와 글리코겐 정량을 하였다. 인공정화의 효과는 FDA National Shellfish Sanitation Program (NSSP)에서 제시한 균 감소율 3.52 Log 이하로 떨어질 때까지 시간을 잡았다. 생굴에 농축된 *V. vulnificus*의 인공정화에 의한 균수 확인은 MPN-PCR을 이용하고 일반세균수분석도 함께 실시하였다. 결과적으로 생굴에서 *V. vulnificus*의 생물농축은 6시간 이후에 최고치를 보였고 이후 유의적인 변화가 없는 것을 확인할 수 있었다. 인공정화에서는 균을 오염시키지 않은 생굴에서 *V. vulnificus*이 검출되지 않았었으며, *V. vulnificus*을 오염시킨 굴에서는 60시간 이상 정화시켰을 때 NSSP 가이드라인에서 제시하는 3.52 Log 저감화가 된 것을 확인 할 수 있었다. 또한, 인공정화에 의한 생굴의 글리코겐 함량과 pH에도 큰 변화는 없어 생굴의 품질변화도 거의 없는 것으로 분석되었다. 이상의 결과는 패류에 대한 안전성 확보를 위하여 인공정화의 효과를 시험한 것으로, 인공정화 체계가 잡히지 않은 우리나라에서

향후 산업적으로 이용 할 수 있는 패류정화 시스템의 개발에 도움이 될 것으로 사료된다.

1. Introduction

Shellfishes mainly growing in coastal area are easily to be contaminated by various fecal matters such as in residential areas, livestock breeding grounds, wildlife habitats, and dock of vessel. As the results, shellfishes harvested in polluted shellfish growing area would contaminate various contaminates including pathogenic microbes and toxic chemicals such as heavy metals (Chiara et al., 2013). Thus, the consumption of contaminated shellfish has been associated many of foodborne disease originated from human and animal fecal contamination such as Salmonella infection and norovirus infection (Rober, 2012). In addition, a shellfish, especially oyster, often causes a severe vibrio infection such as vibrio sepsis due to the consumption of raw oyster contaminated by *Vibrio vulnificus*.

V. vulnificus is a marine bacterium associated with foodborne illness and sepsis. This bacterium causes foodborne illnesses such as vomiting, diarrhea and abdominal pain when eating fish or shellfish contaminated with *V. vulnificus*. However, *V. vulnificus* infection often causes fatal disease such as sepsis in people with chronic liver disease or immunocompromised people or wounded skin infections (Klontz et al., 1988). These reasons, *V. vulnificus* is one of pathogenic bacteria concerned by public health agency (Hlady and Klontz, 1996).

Pathogenic microbes can be concentrated in shellfish internal organ due to the characteristics of filter feeder. A heat treatment is a useful method to prevent foodborne disease caused by pathogenic microbes including bacterial and viral pathogens when taking shellfish products. However, in case of oyster consumption, people often eat by raw without heat treatment, resulting many case of foodborne disease associated with consumption of contaminated oysters (Robert, 1997). To prevent the foodborne disease by contaminated oysters, several strategies have been proposed i) sanitation control in shellfish growing area such as US FDA National Shellfish Sanitation Programs in production stage, ii) relaying contaminated shellfish in clean area until to meet guideline or regulation (FDA, 2017) after harvesting, or iii) depuration contaminated shellfish using clean water in water tank (Lee et al., 2008). Among them, 12 countries including China, France, Ireland, Italy, Malaysia and the United Kingdom are carrying out depuration. Relaying is also practiced in some countries. Relaying involves the transfer of harvested animals to cleaner estuaries or inlets for self-purification in the natural environment. This process can be used as an alternative to depuration for lightly polluted shellfish. Shellfish can only be held for relatively short periods in depuration tanks but can obviously be maintained for much longer periods in the natural environment. The main disadvantages of relaying are the limited availability of suitable unutilized clean coastal areas and of obtaining ownership rights to those areas, the difficulty of controlling water quality and other water parameters and the susceptibility of stock to poaching (Lees et al., 2010).

In case of *Escherichia coli*, it was defined as less than 230 MPN/100g based on the microorganism of the shellfish that could be consumed as raw material (Lee et al., 2008). In case of shellfish exceeding the reference values, natural relaying or depuration measures were taken. In the USA and EU, shellfish harvested in contaminated water have been sold through these purification processes for a long period in order to ensure hygienic safety against microorganism causing bivalve shellfish-associated illness (Catherine et al., 2017). Depuration methods using sterilized seawater have been used in many fields such as oyster, widely used for bivalves (Lees et al., 2010). In Spain, Netherlands and Portugal, the depuration stage of the oyster process control was very important and 75-100% of the oyster production before being marketed. Australia, Ireland, the United States and China were using depuration about 25-50% of their production. In this way, overseas countries have been using depuration (FAO, 2018). However, in Republic of Korea, no specific regulation and guidelines on shellfish relaying and depuration are available for contaminated shellfish.

The object of this study is to evaluate the possibility of depuration of oyster contaminated by *V. vulnificus* that causes fatal sepsis. To progress this study, it firstly performed to prepare the suitable oyster samples. Thus, in this study, there are two main categories. One is the preparation of standard oyster samples contaminated by *V. vulnificus* using bioaccumulation while previous studies on the control or reduction of pathogenic microbes contaminated in shellfish had been used pre-treated samples such as grinded or paste. These samples are not suitable as a model to evaluate the real behaviors of microbes in shellfish samples. Nest is to investigate the usefulness of depuration combined with ultraviolet (UV) treatment in purifying oysters contaminated *V. vulnificus* (Roberta et al., 2012). In addition, it was reported the preparation of standard oyster samples contaminated by *V. vulnificus* using bioaccumulation to evaluate the effectiveness of depuration while previous studies on the control or reduction of pathogenic microbes contaminated in shellfish had been used pre-treated samples such as grinded or paste. In addition, there were no reports on the studies of alive oyster as samples (Oh et al., 2012).

2. Materials and Methods

2.1 Bacterial cultures preparation

Bacterial strain used in this study was *Vibrio vulnificus* KCCM 41665 strain obtained from the Korean Culture Center of Microorganisms (KCCM), Seoul, Korea. This strain was cultivated in Luria-Bertani broth (LB; Difco, Detroit) at 35℃ for 12 h.

2.2 Sample preparation

Crassostrea gigas used in this study is common and industrially important species of oyster. Alive oyster was purchased from an aquaculture farm located at Toyong city, Gyeongnam, Republic of Korea. Oysters were sampled from November 2018 to February 2019 and transported to laboratory under keeping at less than 10℃ within 4 h.

Oysters were selected with weight of 49.3 ± 5.4 g, length of 9.7 ± 1.2 cm, and width of 4.9 ± 2.4 cm. The oysters were washed briefly with tap water to remove mud on shell and placed in a rectangular high-density polyethylene (HDPE) tank (50× 55× 60 cm) containing 100 L of artificial seawater (ASW, salinity; 35 psu) at 15℃ for 14-16 h before being inoculated with *V. vulnificus* to remove debris from inside the oysters. The ASW was prepared by dissolving 1:1 L of artificial salt (Reef Salt Mix; KENT Marine, California) and sea salt (Hanju Salt., Korea) in tap water.

Sixty oysters were transferred to an HDPE tank containing 100 L of freshly prepared ASW containing *V. vulnificus* culture at levels of approximately 10^3or 10^6 CFU/g for each condition. The ASW in HDPE tank was kept temperature at 15℃ using water cooler. Spray bar was used to keep dissolved oxygen (DO) levels for oysters pumping.

2.3 Depuration system

The depuration system tank was made by modifying the conditions of the small-scale shellfish purification proposed by FAO (FAO, 2008). ASW for depuration was recirculating flow rate 480 L/h (8 L/min) to ensure sufficient dissolved oxygen in water. The oysters were placed on a perforated plastic grill that was suspended 90 mm above the bottom of the tank to minimize recontamination (Fig. 1).

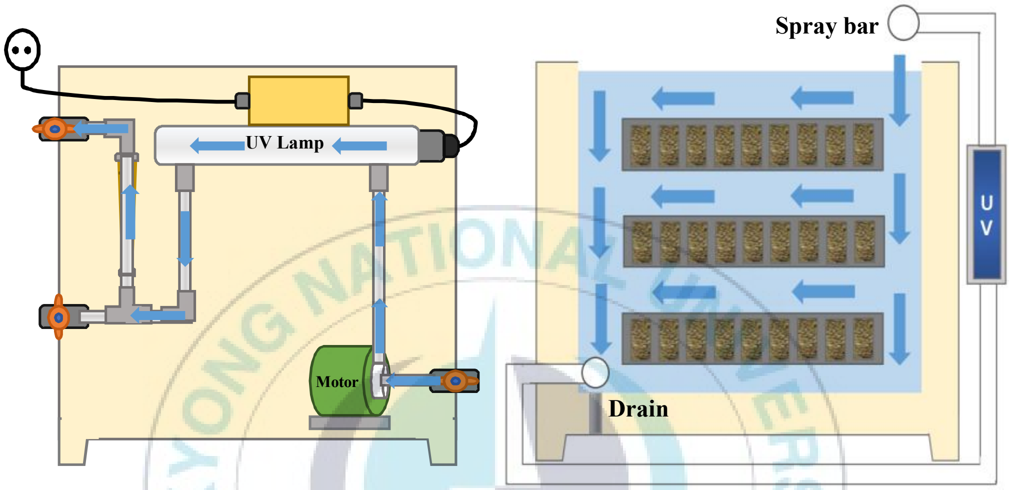

Fig. 1. Diagram of seawater flow through a loaded tank in a recirculation system. The tank containing 100 L of seawater is the size of 50× 55× 60 cm.

2.3.1 Bioaccumulation of *V. vulnificus* into oysters

The ASW was contaminated with the *V. vulnificus* of 10^3 or 10^6 CFU/mL. The air was pumped into the tank to keep the dissolved oxygen levels favorable or oyster pumping, uptake, and bioaccumulation of *V. vulnificus* for a period of 6h. After this period, the oysters were removed from the tank. All bioaccumulated oysters were placed in the depuration tank containing 100 liters of filtered and UV-sterilized ASW. The same experiment was repeated with uncontaminated condition for control.

2.3.2 Depuration combined with UV irradiation

The UV systems consist of a UV tube, filled with gas and mercury, and housed inside a quartz sleeve. Water (8 L/min) is passed parallel to the tube within a jacket of stainless steel or polyvinyl chloride (PVC). Low-pressure mercury vapor lamps produce 85% of their light in the UV-C range at 253.7 nm, which is the wavelength at which peak germicidal activity of UV light occurs (Herrington, 1991). Cell population of *V. vulnificus* in oysters was analyzed during the depuration process time (120 h).

2.4 Microbiological analysis

After the depuration procedure, the oyster shells were cleaned and brushed to remove debris and were then opened aseptically using a sterile shucking knife. The flesh and intervalve liquid of six oysters (about 50 g) was aseptically transferred to sterile bags and homogenized using in a stomacher (Bag Mixer 400VW; Interscience Inc., France) for 2 min by adding about 450 mL of 0.1 M phosphate-buffered saline (PBS; pH 7.0) containing 2% NaCl. The cell population was determined with serial dilution by total viable cell count or most probable number (MPN)-mediated with polymerase chain reaction (PCR) method.

2.4.1 Standard plate count method to enumerate total viable cell in oysters

Total viable count of homogenized oyster mixture and serially diluted mixtures was enumerated by modified standard plate count (SPC) method using Luria-Bertani agar containing 2% NaCl (LB-Na agar). All agar plates were incubated at 35℃ for 18 to 24 h.

2.4.2 MPN-PCR method to enumerate *V. vulnificus* in oysters

Population of *V. vulnificus* in oysters was analyzed by the slightly modified 3-tube most probable number-polymerase chain reaction (MPN-PCR) method according to the U.S. Food and Drug Administration's BAM (FDA, 1998). The homogenized oyster mixtures and serially diluted mixtures were individually inoculated into 3-tubes of alkaline peptone water (APW; pH 8.4, Difco) and incubated at 35℃ for 12 ± 2 h. One loopful of enriched APW culture from a turbid tube was streaked onto individual thiosulfatecitrate-bile salts-sucrose agar (TCBS; Difco) for *V. vulnificus* detection. All plates were incubated at 35℃ for 18 to 24 h. Formation of colonies that were round and green on TCBS was considered positive for *V. vulnificus*. PCR was performed to identify the *V. vulnificus* positive bands and the cell population of *V. vulnificus* was determined by the MPN sheet (FDA, 1998). Primer designs and PCR conditions for specific *V. vulnificus* amplification were performed according to the report (Gitika et al., 2004). The specific primer of *V. vulnificus* were as follows: sense: 5'-CAGCCGGACGTCGTCCATTTTG-3'; antisense: 5'-ATGAGTAAGCGTC CGACGCGT-3 '). PCR was performed at 94℃ for 5 min, denaturation at 94℃ for 30 sec, annealing at 60℃ for 30 sec, elongation at 72℃ for 30 sec, and final elongation at 72℃ for 10 min (Jang et al., 2018).

2.5 Oyster freshness analysis

2.5.1 pH

Changes of pH in oyster during depuration were prepared by mixing shucked-oyster and distilled water at a ratio of 1:10 and measured with a pH meter (Orion 3 star; Thermo Scientific Inc., Fort Collins, Colorado).

2.5.2 Glycogen content

Change of glycogen content in oyster during depuration was determined by the method (Click and Engin, 2005). Shucked oyster samples (0.5 g) were mixed with 5 mL of 30% KOH (Daejung, Shiheung, Korea), sequentially boiled at 95℃ for 20 min, added with 0.5 mL of saturated Na_2SO_4 (Daejung) solution and 5 mL of ethanol(Sigma-Aldrich, St. Louis, Missouri), boiled for 15 min at 95℃, and centrifuged at 1,259 ×g for 10 min. After centrifugation, the precipitate was added with 2 mL of deionized water and 2.5 mL of ethanol (Sigma-Aldrich), centrifuged at 1,259 ×g for 10 min, added 2 mL of 5 M HCl (Samchun, Pyeongtaek, Korea), dissolved completely, and neutralized with 0.5 M NaOH (Samchun). The final neutralized samples (10 mL) was mixed with 5 mL of 0.2% anthron-sulfate solution (Sigma-Aldrich). The mixture was then cooled at 95℃ for 10 min and the absorbance was measured at 620 nm using the spectrophotometer (UV mini-1240; Shimadzu, Japan). Glycogen content was determined by calibration curves of glucose as the standard material and multiplied by the glycogen conversion factor of 0.9.

2.6 Statistical analysis

Analyses were performed in triplicate, and data were averaged over the three measurements. The standard deviation was also calculated. Multiple comparisons were evaluated by two-way analysis of variance using SPSS ver. 25.0 statistical software (SPSS Inc., Chicago). Significant differences between means were determined using Duncan`s multiple range tests. A $p<0.05$ was considered significant.

3. Results and Discussion

3.1 Changes of *V. vulnificus* cell population in oysters during bioaccumulation

To prepare oyster sample contaminated *V. vulnificus* cells using the bioaccumulation, it was tried two different initial inoculums into oyster tank with low-level cell population (10^3 CFU/mL into 100 L seawater tank) or high-level cell population (10^6 CFU/mL into 100 L seawater tank).

In case of high-level intimal inoculation of *V. vulnificus* cells with 10^6 CFU/mL, the cell populations of *V. vulnificus* in oysters rapidly increased and reached at >8.0 log MPN/100g after 6 h of bioaccumulation. Then, a high level of cell population maintained since 6 h of bioaccumulation. In case of low-level intimal inoculation of *V. vulnificus* cells with 10^3 CFU/mL (Fig. 2). Then, a low level of cell population of *V. vulnificus* in oysters reached at >5.0 log MPN/100g after 6 h of bioaccumulation.

However, *V. vulnificus* was not detected in alive oyster before bioaccumulation (Fig. 2). Chae (2009) reported that *Vibrio* spp. rarely detected in seawater below 15℃ of seawater temperature (Chae et al., 2009).

Thus, the oyster samples were not naturally contaminated by *V. vulnificus* since the seawater temperature was bellow than 15℃ at the harvested season (average temperature range in 8-10℃ from November 2018 to February 2019). As shown in Fig. 2, *V. vulnificus* is successfully bioaccumulated in the oyster inside and the cell population tended to be maintained thereafter during bioaccumulation. These results were consistent with the previous reports that bacteria accumulate in shellfish by bioaccumulation (Kwon et al., 2011; Correa et al., 2007). Dore and Lees (1995) also reported that the initial bacterial population rapidly increased and maintained continuously the bacterial population for a short time (1-3 weeks) and a long period (6 months) without relaying or depuration (Dore and Lees, 1995).

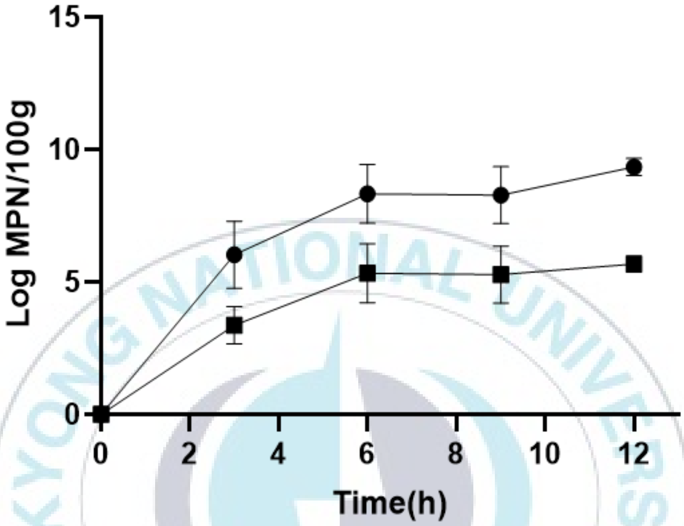

Fig. 2. Changes of *Vibrio vulnificus* cell number (log MPN/100g) in oyster during bioaccumulation at different cell population. (●) Oysters bioaccumulated in water tank inoculated with 10^6 CFU/mL of *V. vulnificus*, (■) Oysters bioaccumulated in water tank inoculated with 10^3 CFU/mL.

3.2 Changes of total viable cell count in oysters during bioaccumulation

As shown in Fig. 3, the number of initial viable cells in the non-bioaccumulated oysters was 2.01-2.46 log CFU/g and the viable cell count increased as progressed the bioaccumulation. The highest viable cell count was observed at 12 h of bioaccumulation with 8.54 log CFU/g in oyster samples with the bioaccumulation of high cell number of *V. vulnificus* (10^6 CFU/mL into 100 L seawater tank) (Fig. 3). In oysters with the bioaccumulation of high cell number of *V. vulnificus* (10^3 CFU/mL into 100 L seawater tank), the viable cells were 5.26 log CFU/g (Fig. 3).

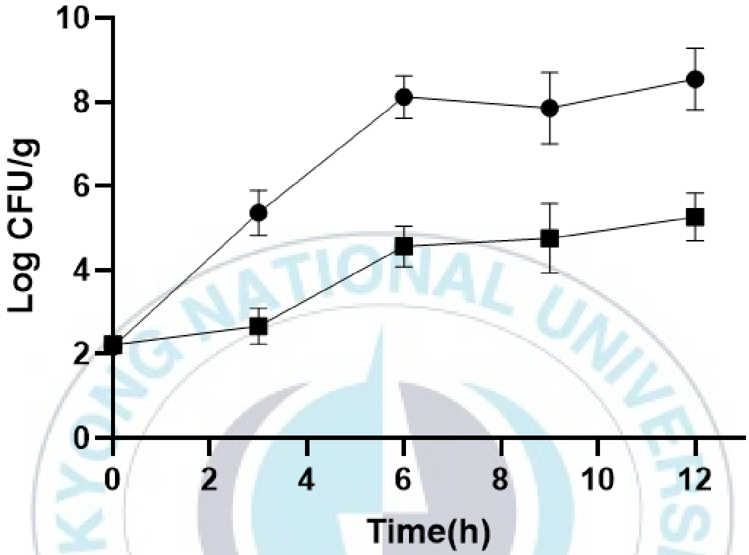

Fig. 3. Changes of total viable cell number (log CFU/g) in oyster during bioaccumulation at different cell population. (●) Oysters bioaccumulated in water tank inoculated with 10^6 CFU/mL of *V. vulnificus*, (■) Oysters **bioaccumulated in water tank inoculated with 10^3 CFU/mL.**

3.3 Reduction of *V. vulnificus* cell population by depuration combined with UV irradiation

No *V. vulnificus* was detected in the non-bioaccumulated oysters during the depuration and no significant changes in viable cell count was observed (Fig. 4, 5). In the oysters bioaccumulated with 8.59 log MPN/100g, the cell number of *V. vulnificus* was significantly decreased as progressed the depuration (Fig. 4). In the case of oyster samples contaminated with low number of *V. vulnificus*, the initial cell number of *V. vulnificus* was to be 4.52 log MPN/100g and showed a tendency to decrease as progressed the depuration (Fig. 4).

According to the NSSP Molluscan Shellfish Control Guideline, the reduction of <3.52 log by a depuration process is considered to be effective (FDA, 2017). Thus, the effective depuration was observed after 60 h of treatment in this study when oysters contaminated with high number of *V. vulnificus* used. After the depuration of 60 h, the cell numbers of *V. vulnificus* were not significantly decreased as reported by Dore and Lees (1995). They also reported that oysters contaminated by *Escherichia coli* were purified more than 98% in 48 h and mussels were purified in 12 h by the depuration combined with UV sterilization (Dore and Lees, 1995).

In contrary to the oysters bioaccumulated with high number of *V. vulnificus*, it was very difficult to evaluate the effectiveness of depuration since the number of *V. vulnificus* bioaccumulated in oysters (4.52 log MPN/100g) was close to the 3.52 log MPN that the reduction point of depuration efficiency suggested by NSSP guideline (FDA, 2017). As shown in Fig. 4, oysters of low-number bioaccumulated with *V. vulnificus* showed 1.66 log MPN/100g of *V. vulnificus* after 21 h of depuration, suggesting that *V. vulnificus* were reduced by the depuration. No significant changes in the cell number of *V. vulnificus* was observed after 21 h of the depuration. Thus, the proposed depuration system equipped with UV sterilization in this study is useful to purified oysters contaminated by *V. vulnificus*.

In this study, it was investigated the possibility of a depuration combined with UV sterilization for oyster samples contaminated by *V. vulnificus*. Considering the above results, it suggests that oyster samples bioaccumulated with high cell number of *V. vulnificus* are a suitable standard shellfish sample to evaluate the efficacy of depuration when oysters contaminate by *V. vulnificus*.

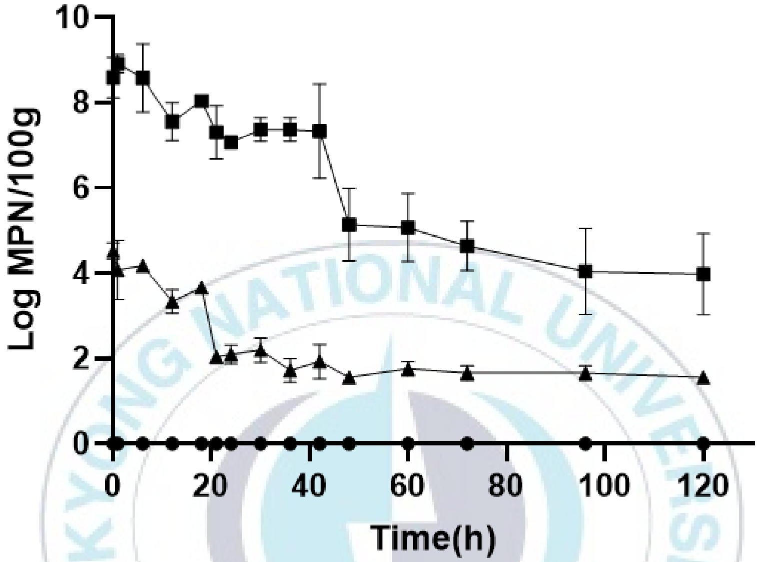

Fig. 4. Changes of *Vibrio vulnificus* cell number (log MPN/100g) in oyster during depuration at different cell population. (●) No bioaccumulated oyster, (■) Oysters bioaccumulated in water tank inoculated with 10^6 CFU/mL of *V. vulnificus*, (▲) Oysters with 10^3 CFU/mL of *V. vulnificus*.

3.4 Changes of total viable cell count in oysters during depuration

The total viable cells in bioaccumulated oysters also decreased as progressed the depuration (Fig. 5). The lowest viable cell count was observed at 72 h of depuration with 3.43 log CFU/g in oyster samples with the bioaccumulation of high cell number of *V. vulnificus* (10^6 CFU/mL into 100 L seawater tank) (Fig. 3). After 72 h of depuration, no significant changes in viable cell counts ranging 3.43 to 3.67 log CFU/g were observed over the periods of depuration (Fig. 5). In case of oysters bioaccumulated with low cell number of *V. vulnificus* (10^3 CFU/mL into 100 L seawater tank), the viable cells were observed with 3.83 log CFU/g at 72 h of depuration. It was also observed no significant changes in viable cell counts ranging 3.83 to 4.15 log CFU/g since 72 h of depuration (Fig. 5). In no bioaccumulated control oysters, no significant changes in viable cell counts ranging 3.04 to 4.59 CFU/g were observed over the periods of depuration. The total viable cell counts in oysters were similar to the MPN results of *V. vulnificus* during the depuration (Fig. 4). These results strongly suggested that no cross contamination was occurred during depuration since the suppling water for the depuration was effectively sterilized by UV irradiation in the depuration tank system.

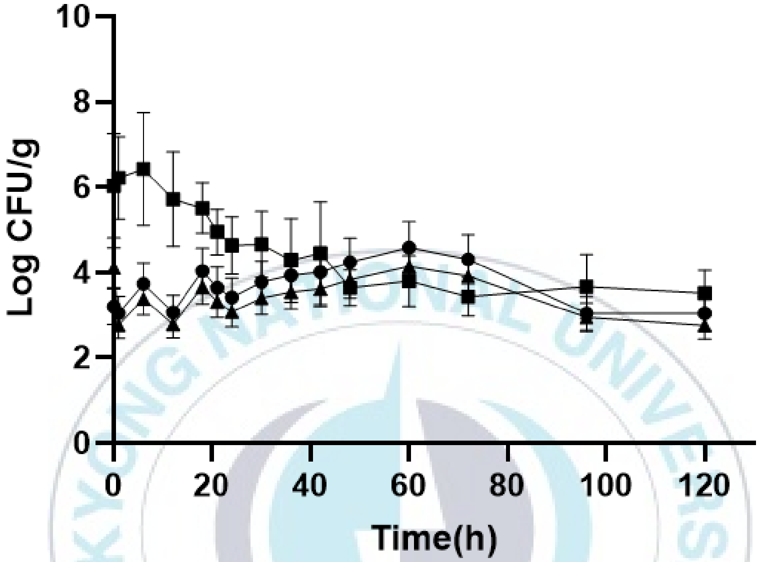

Fig. 5. Changes of total viable cell counts (log CFU/g) in oyster during depuration at different cell population. (●) No bioaccumulated oysters, (■) Oysters bioaccumulated in water tank inoculated with 10^6 CFU/mL of *Vibrio vulnificus*, (▲) Oysters with 10^3 CFU/mL of *V. vulnificus*.

3.5 Change of oyster freshness during depuration combined with UV irradiation

The various complex factors such as physiological factors, individual differences in shellfish, turbidity of seawater, salinity, water temperature, immersion depth, and dissolved oxygen are influential factors for depuration of shellfish (Roderick and Schneider, 1994). In addition, the depuration process will decrease the qualities of oysters since it is working as a stress to shellfish (Metcalf, 1982). Therefore, it was investigated the effect of depuration on the quality of oyster, specially freshness.

3.5.1 Changes of pH in oysters during depuration

pH is an important factor to determine the freshness of oysters (Pottinger, 1948; Hunter and Linden, 1923; Kwon et al., 2011). Also, pH is an important parameter determining quality during oyster storage and distribution. Oysters contains high amount of glycogen that undergoes glycolysis to produce lactate. As the results, the pH decreases after harvest, and during storage and distribution (Cao et al., 2009; Park et al., 2006).

As shown in Fig. 6, no significant pH changes were observed during the depuration. Also, there are no significant difference between oyster samples with or without the treatment of bioaccumulation. The pH range was ranging in pH 6.47 to 6.66 in non-bioaccumulated oyster, pH 6.51 to 6.62 in accumulated with high-cell number, and pH 6.56 to 6.63 in low-number, respectively (Fig. 6). Thus the pH of the oysters was maintained at a constant level regardless of depuration and bioaccumulation of *V. vulnificus*. In addition, it reported that the pH range of fresh oysters is pH 6.3 to 6.5 (Jeong et al., 2015; Son et al., 2014). It has been reported that pH is useful parameter to determine a freshness of oyster. The pH of oyster was 6.3 or higher, "Very good", 6.2 - 5.9 was "good", 5.8 was "off", 5.7 - 5.5 was "musty”, 5.2 or less was judged as "sour" or "putrid" (Pottinger, 1948; Hunter and Linden, 1923). Considering the above results, it suggests that the freshness of oyster was not effect by the depuration.

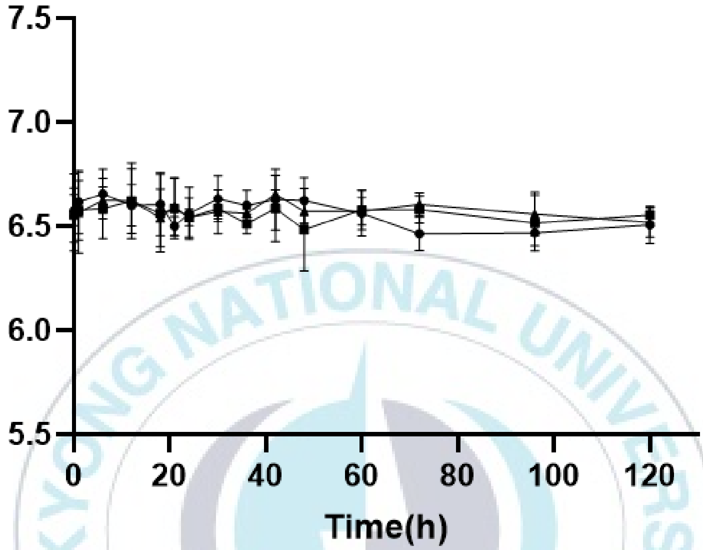

Fig. 6. Changes of pH in oyster during depuration at different cell population. (●) No bioaccumulated oyster, (■) Oysters bioaccumulated in water tank inoculated with 10^6 CFU/mL of *Vibrio vulnificus*, (▲) Oysters with 10^3 CFU/mL of *V. vulnificus*.

3.5.2 Changes of glycogen content in oysters during depuration

As described above, glycogen is the most abundant carbohydrates in oysters and an important factor to determine the freshness and food qualities of oysters (Pottinger, 1948; Hunter and Linden, 1923; Son et al., 2014). Therefore, it was investigated the changes of glycogen contents over the periods of depuration.

As shown in Fig. 7, no significant changes of glycogen content were observed during the depuration. The ranges of glycogen content were in 1.08 to 1.09 g/100g in non-bioaccumulated oyster, 1.08 to 1.12 g/100g in accumulated with high-cell number of *V. vulnificus*, and 1.08 to 1.10 g/100g in low-number of *V. vulnificus*, respectively (Fig. 7). Thus, the changes of glycogen content were similar to the change of pH in oyster during depuration as expected. These results suggest that the bioaccumulation of *V. vulnificus* and depuration were not effect of freshness and qualities of oysters. These results were consistent with the previous reports that the depuration was not affect the glycogen content of oysters (Bob and Richard, 1971)

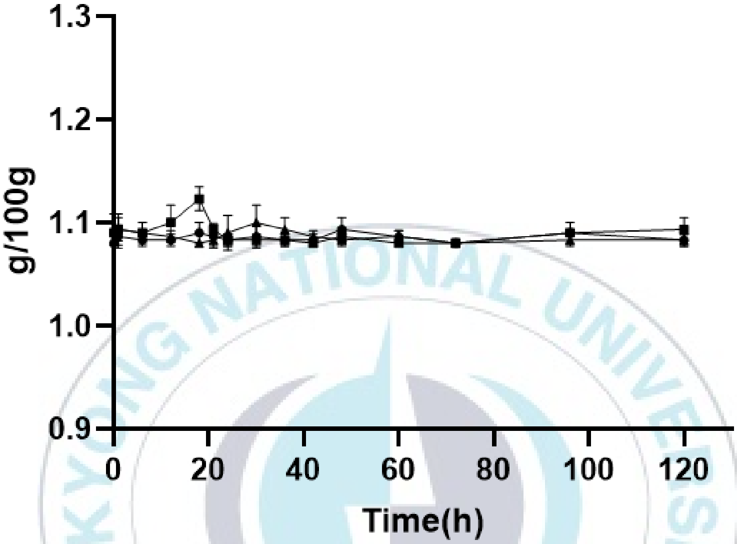

Fig. 7. Changes of glycogen content (g/100g) in oyster during depuration at different cell population. (●) No bioaccumulated oyster, (■) Oysters bioaccumulated in water tank inoculated with 10^6 CFU/mL of *Vibrio vulnificus*, (▲) Oysters with 10^3 CFU/mL of *V. vulnificus*.

4. Conclusion

In order to evaluate the depuration efficacy on oysters contaminated *V. vulnificus*, in this study, it was tried to develop a standard alive oyster samples using a bioaccumulation method. Oysters were bioaccumulated in water tank inoculated with 10^6 CFU/mL of *V. vulnificus*. The cell number of *V. vulnificus* reached at >8.0 log MPN/100g after 6 h of bioaccumulation. Then, the cell population was maintained at a high level since 6 h of bioaccumulation. The oyster samples bioaccumulated by *V. vulnificus* with >8.0 log MPN/100g were used to evaluate the efficacy of depuration combined with UV irradiation. After 60 h of depuration, the cell number of *V. vulnificus* in oysters decreased by <3.52 log, that the reduction point of depuration efficiency suggested by NSSP guideline. In addition, no significant changes in pH and glycogen contents, an important factor of freshness and quality, were observed over the periods of depuration. These results strongly suggest that the depuration proposed in this study is very useful to purify oysters contaminated by *V. vulnificus* without losing freshness and quality.

The above results showed that depuration has secured the safety of shellfish produced in contaminated water. Therefore, this finding may contribute to the development of a shellfish purification system that considers the effects of environmental factors such as temperature, salinity and pH.

It is important to recognize that the most effective and reliable approach to control the microbiological contamination of shellfish is to harvest from areas with good water quality. The best practical approach is therefore should be performed by the shellfish sanitation authorities, which include encouragement, promotion and maintenance of excellent water quality in shellfish production areas. Unfortunately, the suitable marine environment for shellfish cultivation worldwide has been degraded due to discharges from human settlements and agricultural activities. UV irradiation is a disinfection method that are being preferably employed in purification systems in several countries, as unlike other methods, it does not alter the physical or chemical properties of seawater.

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