Research Note

Effects of Flash Freezing, Followed by Frozen Storage, on Reducing Vibrio parahaemolyticus in Pacific Raw Oysters (Crassostrea gigas)

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ABSTRACT

This study investigated the effects of flash freezing, followed by frozen storage, on reducing Vibrio parahaemolyticus in Pacific raw oysters. Raw Pacific oysters were inoculated with a five-strain cocktail of V. parahaemolyticus at a total level of approximately $3.5 \times 10^5$ most probable number (MPN) per gram. Inoculated oysters were subjected to an ultralow flash-freezing process (−95.5°C for 12 min) and stored at −10, −20, and −30°C for 6 months. Populations of V. parahaemolyticus in the oysters declined slightly by 0.22 log MPN/g after the freezing process. Subsequent storage of frozen oysters at −10, −20, and −30°C resulted in considerable reductions of V. parahaemolyticus in the oysters. Storing oysters at −10°C was more effective in inactivating V. parahaemolyticus than was storage at −20 or −30°C. Populations of V. parahaemolyticus in the oysters declined by 2.45, 1.71, and 1.45 log MPN/g after 1 month of storage at −10, −20, and −30°C, respectively, and continued to decline during the storage. The levels of V. parahaemolyticus in oysters were reduced by 4.55, 4.13, and 2.53 log MPN/g after 6 months of storage at −10, −20, and −30°C, respectively. Three process validations, each separated by 1 week and conducted according to the National Shellfish Sanitation Program’s postharvest processing validation–verification interim guidance for Vibrio vulnificus and Vibrio parahaemolyticus, confirmed that a process of flash freezing, followed by storage at $-21 \pm 2^\circ C$ for 5 months, was capable of achieving greater than 3.52-log (MPN/g) reductions of V. parahaemolyticus in half-shell Pacific oysters.

Vibrio parahaemolyticus is a well-documented causative agent of acute human gastroenteritis associated with ingestion of raw or undercooked shellfish and is recognized as a major cause of diarrhea associated with seafood consumption in the United States (2, 8, 11). The Centers for Disease Control and Prevention estimates 4,500 cases of V. parahaemolyticus infection occur each year in the United States (7).

Several outbreaks of V. parahaemolyticus infection associated with raw oyster consumption have been documented in the United States since 1997. Between 1997 and 1998, four major outbreaks of V. parahaemolyticus infections involving more than 700 cases of illness associated with consumption of raw oysters occurred in the Gulf Coast, Pacific Northwest, and Atlantic Northeast regions of the United States (4, 5). In the summer of 2004, 14 passengers on board a cruise ship in Alaska developed gastroenteritis after eating raw oysters produced in Alaska (12). In the summer of 2006, more than 100 people in Oregon, Washington, and British Columbia (Canada) were sickened by V. parahaemolyticus after eating raw oysters harvested from the Puget Sound in Washington and British Columbia. In addition, outbreaks (74 cases) associated with eating raw oysters from the Pacific Northwest were also reported in restaurants and stores of New York City. Between 20 May and 31 July 2006, a total of 177 cases of illness (three hospitalized and no fatalities) were reported in New York City and the states of New York, Oregon, and Washington (6). The number of confirmed cases from the outbreak was greater than was the average number reported between May and July from 2000 to 2004 in the entire United States.

Immediately after the 2006 outbreak, the U.S. Food and Drug Administration (FDA) advised consumers not to consume raw oysters harvested in the Pacific Northwest and forbade sale of oysters from the infected area for raw consumption until the threat was over (17). The often-perceived and occasionally very real threat of V. parahaemolyticus infection after consumption of raw or undercooked oysters is a major concern for public health and causes substantial economic loss to the shellfish industry.

To limit growth of V. parahaemolyticus in raw oysters, the National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish established a time–temperature matrix, limiting the maximum time of exposure of raw oysters to elevated temperatures on harvest. Shellfish harvested for raw consumption needs to be cooled to $10^\circ C$ ($50^\circ F$) within 10, 12, and 36 h of harvest when the average monthly maximum air temperature is $\geq 27^\circ C$ ($\geq 81^\circ F$), be-
between 19 and 27°C (66 and 80°F), and <18°C (<66°F), respectively (14). However, exposure of products to elevated temperatures (>10°C) during retail storage cannot be totally avoided.

Frozen storage is a method commonly used to preserve product quality by inhibiting growth of bacteria and has been reported capable of achieving certain degrees of reductions of *V. parahaemolyticus* in oyster meat. Keeping oysters frozen at −18 and −24°C for 15 to 28 weeks was reported capable of inactivating all the viable cells of *V. parahaemolyticus* (10^5 to 10^7 CFU/g) in oyster homogenates (13). A long-term storage (4 to 6 months) of half-shell Gulf oysters at −20°C was reported capable of reducing low levels of *Vibrio vulnificus* (<1,000 cells per g of oyster) in oysters to undetectable levels (1). Certain oyster producers have utilized the technology to deliver high-quality frozen oysters to consumers for raw consumption. However, no studies have been conducted to determine if frozen storage could be used as a postharvest process to achieve greater than 3.52-log reductions of *V. parahaemolyticus* in oysters, as recommended by the National Shellfish Sanitation Program’s postharvest processing validation–verification interim guidance for *V. vulnificus* and *V. parahaemolyticus* (15).

This study was conducted to determine the efficacy of ultralow (liquid nitrogen gas) freezing process, followed by frozen storage (−10, −20, and −30°C), on inactivating *V. parahaemolyticus* in half-shell Pacific raw oysters.

**MATERIALS AND METHODS**

*Vibrio parahaemolyticus* cultures. Five clinical strains of *V. parahaemolyticus* 10290 (serotype O4:K12), 10292 (serotype O6: K18), 10293 (serotype O1:K56), BE98-2029 (serotype O3:K6), and 027-1C1 (serotype O5:K15) obtained from the FDA’s Pacific Regional Laboratory Northwest (Bothell, WA) were used in this study. Each culture was individually grown in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) supplemented with 1.5% NaCl (overall 2% NaCl) at 37°C overnight (12 to 16 h). The overnight culture was transferred to fresh TSB containing 2% salt and incubated at 37°C for 4 h. The enriched cultures were pooled into a sterile centrifuge tube and harvested by centrifugation at 3,000 × g (Sorvall RC-5B, Kendro Laboratory Products, Newtown, CT) at 5°C for 15 min. Pelleted cells were resuspended in sterile 2% NaCl solution to produce a multistrain cocktail suspension of approximately 10^9 CFU/ml.

**Oyster preparation.** Raw Pacific oysters were obtained from a shellfish farm in Washington and shipped overnight to the laboratory in a cooler with ice gels on harvest. The oysters were briefly washed with tap water to remove mud on the shells and placed in circulating artificial seawater (salinity = 29.6 ppt), prepared by dissolving Instant Ocean Salts (Aquatic Eco-System, Inc., Apopka, FL) in deionized water at room temperature (20 to 23°C) for 3 to 4 h. The oysters were then inoculated with *V. parahaemolyticus* by transferring the oysters to a tank of artificial seawater containing the *V. parahaemolyticus* cocktail at a level of approximately 10^5 CFU/ml, prepared by mixing 1 part of the culture cocktail with 10,000 parts of artificial seawater. Oysters were exposed to *V. parahaemolyticus* in the tank with circulating water (10 liters/h) at 20 to 23°C for approximately 12 to 14 h. Air was pumped into the tank to facilitate colonization of *V. parahaemolyticus* in oysters to a level of approximately 3.5 × 10^5 MPN/g.

**Microbiological analysis.** *V. parahaemolyticus* in oysters was determined with the 3-tube most-probable-number (MPN) procedure described in the FDA’s Bacteriological Analytic Manual online (16). At each testing time, five oysters from each storage temperature were removed from the freezers and allowed to thaw in a refrigerator overnight. Each oyster’s meat was blended with 9 volumes of sterile alkaline peptone water in a sterile blender jar at high speed for 1 min to prepare a 1:10 sample suspension. Two additional 10-fold dilutions of each oyster sample were prepared with sterile alkaline peptone water. All sample dilutions were individually inoculated into three tubes of alkaline peptone salt broth. Inoculated alkaline peptone salt broth tubes were incubated at 35 to 37°C for 16 to 18 h. Each enriched alkaline peptone salt broth showing turbidity after incubation was streaked onto individual thiosulfate–citrate–bile salts–sucrose agar, and the thiosulfate–citrate–bile salts–sucrose agar plates were incubated at 35 to 37°C for 18 to 24 h. Formation of colonies that were round (2- to 3-mm diameter) and green or bluish on thiosulfate–citrate–bile salts–sucrose agar was considered positive for *V. parahaemolyticus*. Total populations of *V. parahaemolyticus* in oysters were determined by converting numbers of alkaline peptone salt broth tubes that were positive for *V. parahaemolyticus* to MPN per gram by using a MPN table. Results were reported as means of five determinations.

For process validation, populations of *V. parahaemolyticus* in oysters were determined with a five-tube MPN procedure. The initial levels of *V. parahaemolyticus* in oysters after inoculation were determined by calculating the arithmetic average of six samples; each consisted of a composite of 10 oysters. Reductions of *V. parahaemolyticus* in oysters during frozen storage were determined each month by analyzing 10 samples; each sample consisted of a composite of 10 oysters. Frozen oysters were moved from the freezer to a refrigerator to allow thawing overnight. Each
TABLE 1. Reductions of Vibrio parahaemolyticus in half-shell Pacific oysters during frozen storage a

<table>
<thead>
<tr>
<th>Storage time (mo)</th>
<th>Temp (°C):</th>
<th>-10</th>
<th>-20</th>
<th>-30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial b</td>
<td></td>
<td>5.49 ± 0.52 A</td>
<td>5.49 ± 0.52 A</td>
<td>5.49 ± 0.52 A</td>
</tr>
<tr>
<td>0 d</td>
<td></td>
<td>5.27 ± 0.26 (0.22) A</td>
<td>5.27 ± 0.26 (0.22) A</td>
<td>5.27 ± 0.26 (0.22) A</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>3.04 ± 0.38 (2.45) B</td>
<td>3.78 ± 0.17 (1.71) B</td>
<td>4.05 ± 0.45 (1.45) B</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>2.68 ± 0.23 (2.81) B</td>
<td>3.16 ± 0.38 (2.34) C</td>
<td>3.64 ± 0.26 (1.85) B C</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>2.16 ± 0.31 (3.34) C</td>
<td>2.66 ± 0.36 (2.83) C D</td>
<td>3.33 ± 0.24 (2.16) C D</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1.67 ± 0.72 (3.83) C D</td>
<td>2.35 ± 0.16 (3.14) D</td>
<td>3.22 ± 0.62 (2.28) D</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>1.49 ± 0.28 (4.01) D</td>
<td>1.86 ± 0.19 (3.63) E</td>
<td>3.15 ± 0.49 (2.35) D</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.94 ± 0.07 (4.55) D</td>
<td>1.37 ± 0.15 (4.13) F</td>
<td>2.97 ± 0.34 (2.53) D</td>
</tr>
</tbody>
</table>

a Values are expressed in log MPN per gram, and are means of five determinations ± standard deviations.
b Populations before the freezing process.
c Means with the same letter in the same column are not significantly different (P > 0.05).
d Populations after the freezing process.
*f* Reductions of *V. parahaemolyticus* after treatments.

A composite sample was prepared by pooling meats and liquors of 10 oysters in a sterile blender jar and blending at high speed for 1 min. Twenty-five grams of the homogenized sample was mixed with 225 ml of sterile alkaline peptone water to prepare a 1:10 sample dilution.

**Data analysis.** Results of microbiological tests were transformed into log values for statistical analyses. Populations of *V. parahaemolyticus* in oysters at different storage times within a treatment were analyzed with Turkey-Kramer multiple comparison test (S-Plus, Insightful Corp., Seattle, WA). Significant differences between means of treatments were established at a level of *P* = 0.05.

**RESULTS AND DISCUSSION**

**Effects of flash freezing and frozen storage on *V. parahaemolyticus* in oysters.** Flash freezing that is followed by frozen storage is commonly used to prevent growth of bacteria in foods and preserve quality of products. However, the freezing process itself usually has little effect on inactivating bacteria. In this study, populations of *V. parahaemolyticus* in inoculated oysters declined slightly (0.22 log MPN/g of reduction) after the ultralow, flash-freezing process (Table 1). However, subsequent storage of frozen oysters at −10, −20, and −30°C resulted in considerable declines in *V. parahaemolyticus* counts.

The populations of *V. parahaemolyticus* in the oysters declined much faster during the first month of frozen storage at all three temperatures and continued to decline at a slower rate during storage (Table 1). Populations of *V. parahaemolyticus* in the oysters decreased by 2.45, 1.71, and 1.45 log MPN/g after 1 month of storage at −10, −20, and −30°C, respectively. After 4 months of storage, the levels of *V. parahaemolyticus* in oysters were reduced by 3.83 (−10°C), 3.14 (−20°C), and 2.28 (−30°C) log MPN/g. At the end of 6 months of study, the levels of *V. parahaemolyticus* were reduced by 4.55, 4.13 and 2.53 log MPN/g in oysters stored at −10, −20, and −30°C, respectively.

It is well-known that temperatures of frozen storage can affect the survival of bacteria in food products, and bacteria tend to survive better in products stored at lower temperatures. Therefore, it was not a surprise to observe that *V. parahaemolyticus* survived better in oysters stored at −20 or −30°C than at −10°C. This is mainly because intracellular ice crystals formed in bacterial cells at lower temperatures (−20 or −30°C) are smaller than those formed at a higher temperature such as −10°C during frozen storage. Thus, bacteria tend to survive better at lower freezing temperatures due to less cell damage (disruption of cell membrane, cell wall, and internal structure) caused by ice crystals (9). A previous study of survival of several strains of *V. parahaemolyticus* in shrimp meat homogenates at −20 and −80°C reported that the bacterial counts remained almost unchanged in the homogenate stored at −80°C after an initial 1-log reduction of the bacterial counts (3). However, populations of *V. parahaemolyticus* in shrimp meat homogenates were reduced by approximately 5 log after 25 days of storage at −20°C. Another study also reported that mortality rate of *V. parahaemolyticus* in shucked oysters stored at −15°C was higher than that of oysters stored at −30°C (10).

**Process validation.** While the frozen study indicated that a process of flash freezing followed by frozen storage at −10°C for 4 months or at −20°C for 5 months could result in >3.52-log reduction of *V. parahaemolyticus* in half-shell Pacific oysters, the process needs to be validated according to the National Shellfish Sanitation Program’s Guide for the Control of Molluscan Shellfish (15). It seems that storing oysters at −10°C may have advantages over the storage at −20 or −30°C because of less energy usage and greater reductions of *V. parahaemolyticus* in oysters. However, holding oysters at 21 ± 2°C has been used by the shellfish industry for long-term storage (3 to 6 months) of raw oysters before shipping the oysters to consumers. Therefore, the process validation was conducted with storage at −21 ± 2°C.

The reductions of *V. parahaemolyticus* in Pacific half-shell oysters stored at −21 ± 2°C are shown in Table 2. Similar to the initial study, populations of *V. parahaemolyticus* in the oysters declined more rapidly during the first month of frozen storage (1.5 to 2.6 log MPN/g) and continued to decline at a slower rate during the storage. The
TABLE 2. Process validation of frozen storage at −21 ± 2°C for reducing Vibrio parahaemolyticus in half-shell Pacific oysters

<table>
<thead>
<tr>
<th>Storage time (mo)</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initialb</td>
<td>5.57 ± 0.54</td>
<td>5.13 ± 0.55</td>
<td>5.09 ± 0.56</td>
</tr>
<tr>
<td>0c</td>
<td>5.21 ± 0.39 (0.36) A</td>
<td>3.49 ± 0.26 (1.64) A</td>
<td>4.48 ± 0.54 (0.61) A</td>
</tr>
<tr>
<td>1</td>
<td>4.04 ± 0.32 (1.53) B</td>
<td>2.57 ± 0.36 (2.56) B</td>
<td>3.33 ± 0.38 (1.76) B</td>
</tr>
<tr>
<td>2</td>
<td>2.62 ± 0.26 (2.95) C</td>
<td>1.93 ± 0.33 (3.20) C</td>
<td>2.63 ± 0.22 (2.73) C</td>
</tr>
<tr>
<td>3</td>
<td>2.32 ± 0.36 (3.25) D</td>
<td>1.52 ± 0.19 (3.61) D</td>
<td>2.05 ± 0.42 (3.04) CD</td>
</tr>
<tr>
<td>4</td>
<td>2.17 ± 0.24 (3.40) D</td>
<td>1.69 ± 0.24 (3.44) D</td>
<td>1.68 ± 0.37 (3.41) D</td>
</tr>
<tr>
<td>5</td>
<td>1.81 ± 0.48 (3.76) E</td>
<td>0.61 ± 0.34 (4.52) E</td>
<td>0.87 ± 0.43 (4.22) E</td>
</tr>
<tr>
<td>6</td>
<td>1.25 ± 0.17 (4.32) F</td>
<td>0.61 ± 0.24 (4.52) E</td>
<td>0.55 ± 0.22 (4.54) E</td>
</tr>
</tbody>
</table>

a Values are expressed in log MPN per gram, and are means of 10 determinations (10 samples, each composed of 10 oysters) ± standard deviations.
b Populations before the freezing process.
c Populations after the freezing process.
d Reductions of V. parahaemolyticus after treatments.
e Means with the same letter in the same column are not significantly different (P > 0.05).

reductions of V. parahaemolyticus in oysters increased to ≧3.0 log MPN/g (3.0, 3.3, and 3.6 log MPN/g) after 3 months of storage and to >3.5 log MPN/g (3.8, 4.2 and 4.5 log MPN/g) after 5 months of storage. At the end of 6 months of storage, the reductions of V. parahaemolyticus in oysters increased to 4.3 to 4.5 log MPN/g, with very low levels (<20 MPN/g) of V. parahaemolyticus being detected in the oysters (Table 2).

While several studies have reported reductions of V. parahaemolyticus in shrimp meat homogenates and shucked oysters (3, 10), no studies have been conducted to identify a process involving flash freezing that is followed by frozen storage for reducing V. parahaemolyticus in oysters by a minimum of 3.52 log. This study identified a process of flash freezing (−95.5°C for 12 min) that, followed by storage at −21 ± 2°C for 5 months, could achieve >3.52-log reductions of V. parahaemolyticus in half-shell Pacific oysters. The process was validated according to the National Shellfish Sanitation Program’s Guide for the Control of Molluscan Shellfish (15).

Flash freezing that is followed by frozen storage can be used as a postharvest process for reducing V. parahaemolyticus contamination in half-shell Pacific oysters. A process of flash freezing (−95.5°C for 12 min) that was followed by storage at −21 ± 2°C for 5 months was capable of achieving greater than 3.52-log (MPN/g) reductions of V. parahaemolyticus in half-shell Pacific oysters.

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REFERENCES