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# Effect of Diluent Salt Concentration and pH on the Enumeration of *Vibrio parahaemolyticus* by Direct Plating on Selective Agar

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**Abstract** The maintenance of physiological activity during dilution is very critical for the accurate enumeration of *Vibrio* spp. in marine samples. We investigated the effect of various diluents on the recovery of *Vibrio parahaemolyticus* using the direct plate counting and most probable number (MPN) methods. The effects of NaCl (0.85 and 3%) and pH (from 6.6 to 7.4) in diluents based on distilled water or phosphate buffered saline (PBS) were evaluated with three *V. parahaemolyticus* strains. PBS-3% NaCl (pH 6.6), as opposed to PBS, was the most effective diluent at maintaining viable cell numbers up to 2 log CFU/g during dilution for direct plate counting using on thiosulfate citrate bile salts sucrose (TCBS) selective agar, as well as minimizing the difference in cell numbers between TCBS and non-selective nutrient agar. It also increased counts of *V. parahaemolyticus* inoculated into oysters relative to PBS ($p$<0.01), suggesting that PBS-3% NaCl (pH 6.6) can reduce the problem of underestimating *V. parhaemolyticus* counts using PBS alone.

**Keywords:** *Vibrio parahaemolyticus*, oyster, diluents, direct plate counting

## Introduction

Quantitative microbial risk assessment has become an important part of food safety, and it is necessary to accurately evaluate the level of microorganisms in food at the time of consumption (1, 2). Increasing the detection sensitivity of bacteria in the sample is required to avoid underestimating the actual number of viable cells in food. Therefore, the selection of an appropriate diluent is an essential step in the enumeration of specific bacteria without increasing or decreasing the cell numbers during dilution, especially when their physiological activity is very sensitive to temperature, pH, nutrient concentration, and salinity (3, 4).

*Vibrio parahaemolyticus* is a halophilic rod, Gram negative, and mesophilic foodborne pathogen that causes gastroenteritis with severe abdominal pain and diarrhea. Raw fish and shellfish are the most important sources of foodborne illness caused by *V. parahaemolyticus* (5). *V. parahaemolyticus* is also known to be a major foodborne pathogen in Asia. It is one of the leading causes of foodborne disease in Korea (6-11). *V. parahaemolyticus* outbreaks in Korea occur mainly in August and September with a few cases in May. Therefore, developing a simple and accurate method of counting the number of pathogenic *Vibrio* spp. is necessary to ensure product safety in the seafood industry (5, 12).

To enumerate *V. parahaemolyticus* cells in environmental samples, the Bacteriological Analytical Manual of Food and Drug Administration (13) recommends three methods: most probable number (MPN), hydrophobic grid membrane filter (HGMF), and DNA probes. Among them, MPN has been widely used to detect and quantify *Vibrio* strains from environmental samples because the MPN method contains an enrichment step (14) to resuscitate any injured cells in environmental samples. However, estimates with the MPN method can vary widely from the actual numbers, and it is difficult to get a positive confirmation test from highly diluted samples (15). On the other hand, direct plate counting is a precise and simple method to compare the effects of physical/chemical treatment on populations of *Vibrio* spp. (16, 17). Selective agar has also been used in direct plate counting to facilitate the growth of selected microbial groups present in food.

It has been reported that the multiplication of *Vibrio* spp. is closely related to the temperature, salinity, and pH of seawater (18). Environmental factors such as salinity and pH also affect the adhesion activity (19) and pathogenecity (20) of *Vibrio* strains in seafood, as well as their survival (21, 22). It was reported that increased salinity up to 3% in the diluent was more effective for quantifying *Vibrio* spp. compared to the 0.85% salinity of physiological solutions, or 0.76% phosphate buffered saline (PBS, 23-26). One tenth % peptone water with 3% NaCl at pH 6.6 has also been recommended for plate counting marine species such as *Vibrio vulnificus* (23). However, the degree to which changes in salinity of the diluent can improve the preservation of *V. parahaemolyticus* in environmental samples or buffer systems has not yet been reported. Since the salinity of seawater of South Korea (Gujae, Goheong, and Namhae) changes between 1 and 3.5% throughout the year depending on the season and the amount of rainfall (Fig. 1), it is very important to find the appropriate diluent for the accurate enumeration of *V. parahaemolyticus* in oysters originating from Korean sea water (27).

Therefore, the objective of this study was to investigate

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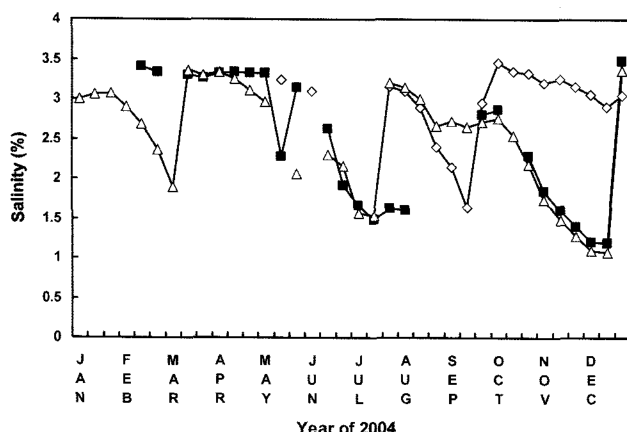


**Fig. 1. Changes in salinity of the South Sea of Korea.** Salinity on the coasts of Gujae (◇), Goheong (■), and Namhae (△) (27).

the effects of salinity and pH on distilled water or PBS to find the most appropriate diluent for the enumeration of *V. parahaemolyticus* by direct plate counting with selective agar and the MPN method.

## Materials and Methods

**Bacterial strains** Three strains of *V. parahaemolyticus* (ATCC 17802, ATCC 27969, and ATCC 33844) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The strains of *V. parahaemolyticus* ATCC 17802, ATCC 27969, and ATCC 33844 were cultured in nutrient broth with 3% NaCl at 37 °C, in trypticase soy agar with 2.5% NaCl at 37°C, and in Marine Agar (Difco Laboratories, Becton Dickinson Co., Sparks, MD, USA) at 30°C, respectively, according to the ATCC manual and preserved at -80°C.

**Preparation of diluents** Salt was added to sterilized distilled water or PBS (7.650 g NaCl, 0.724 g $Na_2HPO_4$ (Sigma, St. Louis, MO, USA), 0.210 g $KH_2PO_4$ in 1 L distilled water, at pH 7.4) to a final concentration of 0, 0.85, or 3.0%. The pH of each diluent was adjusted to 6.6, 6.8, 7.0, 7.2, or 7.4 with the addition of 1 N HCl or 1 N NaOH.

**Dilution and enumeration of *V. parahaemolyticus*** Ten μL of *V. parahaemolyticus* cultures grown overnight at 37°C for 15 hr was inoculated into 10 mL of nutrient broth containing 3% NaCl and incubated at 37°C at 200 rev per min until the $O.D._{600}$ reached 0.5. After vortexing 10 mL of the culture, 1 mL of the culture was centrifuged for 5 min at 10,000×*g* in a microcentrifuge (VS-15000 CFN II; Vision Scientific & Laboratory Instrument, Suwon, Korea). The collected cells were suspended in 1 mL of distilled water or PBS (pH 7.4) containing 0, 0.85, or 3% NaCl, respectively. Each diluent was again serially diluted with pH adjusted distilled water or PBS containing 0, 0.85, or 3 % NaCl, respectively. One tenth mL of each diluent was plated on thiosulfate citrate bile salts sucrose (TCBS) agar or nutrient agar (Difco) with 3% salt in duplicate. After incubation at 37°C for 24 hr, *V. parahaemolyticus* colonies on each plate were counted.

In order to test the ability of each diluent to maintain the viability *V. parahaemolyticus* during dilution, the cells were incubated at room temperature (20-23°C) or 37°C for 0 or 2 hr and plated on TCBS agar in duplicate. All steps of dilution and plating for each sample were performed within 10 min at ambient temperature to avoid losing cell viability.

**Inoculation of *V. parahaemolyticus* in oysters** Cultivated oysters (*Crassostrea gigas*) packed in seawater were purchased from a supermarket in Seoul, Korea and stored for 1 hr at ambient temperature to avoid cold shock of *V. parahaemolyticus* culture during inoculation. Ten oysters were weighed (approximate 60 g) and 0.1 mL of the overnight culture was inoculated into each oyster to prepare at least $10^5$ cells/g using a sterile syringe according to the method of Azanza *et al.* (23).

**Enumeration of *V. parahaemolyticus* in oysters using the selective agar plating and MPN methods** Enumeration of *V. parahameolyticus* in oysters was conducted using the selective agar plating and MPN methods as described in the FDA Bacteriological Analytical Manual (13). Five hundred forty mL of the prepared diluent (PBS at pH 7.4 and PBS-3% NaCl at pH 6.6) was added to 10 oysters and the mixture was blended for 90 sec at maximum speed using a Waring blender (Model 31BL9; Waring Commercial®, New Hartford, CT, USA). For the plating method, 0.1 mL of each diluent was plated on TCBS agar or nutrient agar (Difco) with 3% salt in duplicate. After incubation at 37°C for 24 hr, *V. parahaemolyticus* colonies on each plate were counted.

For the MPN method, oysters were blended for 90 sec as described above and 1:100. Dilutions of 1:1,000, 1: 10,000 dilutions in alkaline peptone water (APW) (10 g peptone, 10 g NaCl, 1 L distilled water) were prepared in separate tubes and incubated overnight at 37°C. The overnight cultures were streaked onto TCBS agar and incubated overnight at 37°C. The green colonies on TCBS agar were transferred to agar containing Cellobiose colistin (CC) (10 g Peptone, 5 g Beef extract, 20 g NaCl), 1 mL 1,000× dye stock solution (4 mg Bromthymol blue, 4 mg Cresol red, 1 mL ethanol), 15 g agar, 900 mL distilled water, 10 g cellobiose (Sigma), and 400,000 units colistin (Sigma), 100 mL distilled water). The colonies which did not grow on CC agar were transferred to gelatine agar (GA) (Difco) and gelatine salt agar (GS) with 3% NaCl. The colonies that grew on GS agar but not on GA agar were tested for oxidase positive and gram negative strains. The suspicious colonies were confirmed by using API 20E diagnostic strips (Biomerieux, Marcy I'Etoile, France). The number of MPN tubes containing confirmed colonies was compared to the 3-tube MPN chart and the results expressed as MPN/g for *V. parahaemolyticus*.

**Statistics** The means ± standard deviations of the viable cell numbers were transformed to logarithmic values. Paired statistical comparison of the logarithmic cell numbers ($\log_{10}N$) between PBS and PBS-3% NaCl (pH 6.6) was performed using the Student's *t*-test (28). Analysis of variance (ANOVA) and Duncan's multiple range tests (29) were conducted using the SAS program

(version 6.12) for comparison of the numbers of *V. parahaemolyticus* cells among the diluents.

## Results and Discussion

Various diluents with the addition of salt have been used for the growth of marine species due to their halophilic characteristics (17, 18, 30). According to the bacteriological analytical manual (BAM) guidelines, PBS is recommended for blending initial isolates of *V. parahaemolyticus* from seafood (13). Addition of 3% NaCl in the diluent has been shown to increase the cell number of viable *Vibrio* spp. present in seafood (23).

In the present study, diluent made with distilled water or PBS at various pH levels with NaCl concentrations of 0, 0.85, or 3% were evaluated to find the optimum diluent conditions for enumerating *V. parahaemolyticus* accurately. *V. parahaemolyticus* (ATCC 17802) samples diluted in distilled water or PBS containing 0, 0.85, or 3% were held for 0 or 2 hr at 37°C prior to plating on TCBS agar and then compared (Table 1). There were no viable counts in distilled water without NaCl although other diluents containing NaCl showed various *V. parahaemolyticus* levels. Diluent containing 3% NaCl, whether based on distilled water or PBS, significantly increased ($p<0.05$) *V. parahaemolyticus* viablility following a 2 hr incubation in diluent (Table 1). The effect of 2 hr incubation prior to plating on the numbers of *V. parahaemolyticus* was not significant for PBS diluents without salt or with 0.85% salt. This result suggests that salt content is a more important factor than diluent base for the recovery of viable *V. parhaemolyticus* in this study.

Table 2 shows the effect of pH on *V. parahaemolyticus* numbers in PBS containing 3% NaCl with incubation times following dilution of either 0 or 2 hr at 37°C. No significant differences in *V. parahaemolyticus* levels were observed with PBS containing 3% NaCl at various pH levels with an incubation of 0 hr. However, *V. parahaemolyticus* levels were significantly ($p<0.05$) increased at pH levels higher than 6.8 after a 2 hr incubation at both ambient temperature (data not shown) and 37°C. This indicates that diluents with pH values above 6.8 improved the recovery

**Table 1. The Effects of diluents and dilution time on *V. parahaemolyticus*[1)] counts by direct plating on TCBS selective agar**

| Diluents | *V. parahaemolyticus* counts (Log $_{10}$CFU/g) on dilution time[2)] | |
|---|---|---|
| | 0 hr | 2 hr |
| Distilled Water (DW) | ND[3)] | ND |
| DW-0.85% NaCl | 7.20±0.03$^{bx}$ | 7.02±0.45$^{ax}$ |
| DW-3% NaCl | 7.72±0.03$^{cx}$ | 8.13±0.12$^{bcy}$ |
| PBS | 7.01±0.12$^{ax}$ | 7.10±0.68$^{ax}$ |
| PBS-0.85% NaCl | 7.14±0.07$^{abx}$ | 7.42±0.03$^{abx}$ |
| PBS-3% NaCl | 8.06±0.03$^{dx}$ | 8.34±0.04$^{cy}$ |

1)*V. parahaemolyticus* ATCC 17802 was used.
2)abMean±SD (n=3) with different letters in the same column are significantly different ($p<0.05$); xyMean±SD (n=3) with different letters in the same row are significantly different ($p<0.05$).
3)ND: not detectable.

**Table 2. The effect of diluent pH (PBS-3% NaCl) on *V. parahaemolyticus*[1)] counts by direct plating on TCBS selective agar**

| PBS-3% NaCl | *V. parahaemolyticus* counts (Log $_{10}$CFU/g) on dilution time at 37°C[2)] | |
|---|---|---|
| | 0 hr | 2 hr |
| pH 6.6 | 7.97±0.10$^{ax}$ | 8.01±0.15$^{ax}$ |
| pH 6.8 | 7.96±0.05$^{ax}$ | 8.38±0.08$^{by}$ |
| pH 7.0 | 7.91±0.06$^{ax}$ | 8.29±0.02$^{by}$ |
| pH 7.2 | 7.81±0.16$^{ax}$ | 8.34±0.10$^{by}$ |
| pH 7.4 | 7.72±0.22$^{ax}$ | 8.30±0.06$^{by}$ |

1)*V. parahaemolyticus* ATCC 17802 was used.
2)abMean±SD (n=3) with different letters in the same column are significantly different ($p<0.05$); xyMean±SD (n=3) with different letters in the same row are significantly different ($p<0.05$).

of *V. parahaemolyticus* in PBS-3% NaCl, and thus increased *V. parahaemolyticus* numbers with an extended dilution period.

Table 3 shows the effect of diluents on the viability of various strains of *V. parahaemolyticus* by direct plating on non-selective nutrient agar and selective TCBS agar. Regardless of the *V. parahaemolyticus* strain, no diluent effect was observed regarding *V. parahaemolyticus* counts on non-selective agar. However, all the strains tested in this study showed significantly higher recovery counts in PBS containing 3% NaCl (about 1-2 log CFU/g) than PBS alone on selective TCBS agar ($p<0.01$). This indicates that diluent is an important factor for increasing the recovery of *V. parahaemolyticus* on selective media. These results show that dilutions of *V. parahaemolyticus* in PBS result in a decrease in *V. parahaemolyticus* viability when they are spread onto selective TCBS agar.

Thus, PBS-3% NaCl reduces the magnitude of the underestimation of *V. parahaemolyticus* counts by the direct plating method with TCBS selective agar. Overall, the numbers of *V. parahaemolyticus* recovered were significantly ($p<0.05$) higher on non-selective nutrient agar than selective TCBS agar for both PBS and PBS-3% NaCl. This was especially true with the *V. parahaemolyticus* ATCC 33844 strain, in which the differences in recovery for *V. parahaemolyticus* between non-selective and selective media were 4.66 logs in PBS and 2.32 logs in PBS-3% NaCl (Table 3). Overall, PBS-3% NaCl reduces the difference of *V. parahaemolyticus* counts between selective TCBS agar and non-selective nutrient agar.

In order to confirm the superiority of PBS with 3% NaCl (pH 6.6) as a diluent for the enumeration of *V. parahaemolyticus* in seafood over PBS alone (pH 7.4), *V. parahaemolyticus* ATCC 17802 was artificially inoculated into oysters. The viable numbers of *V. parahaemolyticus* in oysters were compared between PBS alone (pH 7.4) and PBS-3% NaCl (pH 6.6) on TCBS selective agar (Table 4). PBS-3% NaCl significantly ($p<0.05$) increased the recovery of *V. parahaemolyticus* inoculated into oysters. Azanza *et al.* (23) has shown a similar improvement in *V. vulnificus* enumeration with the addition of 3% NaCl to 0.1% peptone solution. The MPN method from the BAM, PBS is usually used for the initial isolation of *V. parahaemolyticus* from shellfish (13). When PBS-3% NaCl (pH 6.6)

**Table 3. Effect of diluents on the level of three strains of *V. parahaemolyticus* between TCBS selective and nutrient non-selective agar**

| | | *V. parahaemolyticus* counts ($Log_{10}$ CFU/g) in diluents | | |
|---|---|---|---|---|
| Strain | Media | PBS | PBS-3% NaCl (pH 6.6) | *t*-value |
| *V. parahaemolyticus* ATCC 17802 | TCBS agar[1)] | 5.29±0.06 | 6.66±0.10 | -17.91* |
| | Nutrient agar[2)] | 8.05±0.30 | 8.18±0.03 | -5.29 |
| | *t*-value | -63.57* | -17.45* | |
| *V. parahaemolyticus* ATCC 33844 | TCBS agar | 3.51±0.18 | 5.86±0.32 | -15.79* |
| | Nutrient agar | 8.18±0.19 | 8.18±0.04 | 0.01 |
| | *t*-value | -25.05* | -16.32* | |
| *V. parahaemolyticus* ATCC 27969 | TCBS agar | 6.56±0.15 | 7.66±0.06 | -35.31* |
| | Nutrient agar | 8.35±0.14 | 8.45±0.04 | -1.52 |
| | *t*-value | -12.44* | -8.84 | |

[1)]TCBS agar indicates a selective media.
[2)]Nutrient agar contains 3% NaCl and indicates a non-selective media. *$p<0.05$.

**Table 4. Effect of diluents on *V. parahaemolyticus*[1)] counts in oysters by direct plating on TCBS selective agar**

| Diluents | $Log_{10}$ CFU/g | *t*-value | *p*-value |
|---|---|---|---|
| PBS (pH 7.4) | 5.97±0.06[a] | | |
| PBS-3% NaCl (pH 6.6) | 6.87±0.07[b] | -67.26 | $p<0.05$ |

[1)]*V. parahaemolyticus* ATCC 17802 was used in this study.

was used for the 3 tube-MPN method of quantitative detection of *V. parahaemolyticus* in oysters in the present study, the value was 0.8 log MPN/g higher than PBS alone (pH 7.4), which was not significantly ($p<0.05$) different (data not shown). In the MPN method, the homogenate of *V. parahaemolyticus*-oyster-PBS (or PBS-3% NaCl) was quickly diluted in APW and enriched according to the FDA-BAM manual. In direct plate counting, the homogenate of *V. parahaemolyticus*-oyster-PBS (or PBS-3% NaCl) was diluted and maintained longer in PBS (or in PBS-3% NaCl) before being plated compared to the MPN method. Presumably the 90 sec oyster blending time in PBS-3% NaCl instead of PBS is too short to result in a difference in *V. parahaemolyticus* viability.

Ogden *et al.* (31) also compared the direct plating method using selective Chromocult agar to the MPN method and showed the same quantitative results with *Escherichia coli.* It was reported that the MPN method can give less than 1 log unit higher counts of *Vibrio cholera* compared to direct enumeration with TCBS (32). Therefore, the MPN method rather than the direct plate counting is recommended when enumerating *Vibrio* spp. from environmental samples. However, dilution of *V. parahaemolyticus* in PBS-3% NaCl is more appropriate than PBS when the direct plate counting method is used (i.e., comparing the effects of physical/chemical treatments or the effects before and after processing on *V. parahaemolyticus*).

In conclusion, the development of selective media with an increase in the recovery of *V. parahaemolyticus* is needed for the simple and accurate plate counting method. PBS-3% NaCl at pH 6.6 was found to be an appropriate diluent for recovering *V. parahaemolyticus* in both broth and oysters for the direct plate counting method in this study, and thus can be recommended for the enumeration of marine *V. parahaemolyticus* bacteria. The results demonstrate that the use of an appropriate diluent for the direct selective agar method can overcome deficits in the MPN method by improving the quantitative recovery of microorganisms in environmental samples.

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## References

1. Duffy G, Cummins E, Nally P, O'Brien S, Butler F. A review of quantitative microbial risk assessment in the management of *Escherichia coli* O157:H7 on beef. Meat Sci. 74: 76-88 (2006)
2. Nwachuku N, Gerba CP. Microbial risk assessment: don't forget the children. Curr. Opin. Microbiol. 7: 206-209 (2004)
3. Wang Y, Gu JD. Influence of temperature, salinity, and pH on the growth of environmental *Aeromonas* and *Vibiro* species isolated from Mai Po and the Inner Deep Bay Nature Reserve Ramsar Site of Hong Kong. J. Basic Microb. 1: 83-93 (2005)
4. McFeters GA, Cameron SC, LeChevallier MW. Influence of diluents, media, and membrane filters on detection of injured waterborne coliform bacteria. Appl. Environ. Microb. 43: 97-103 (1982)
5. Sakazaki R. *Vibrio.* pp. 127-136. In: Foodborne Diseases. 2nd ed. Cliver DO, Riemann HP (eds). Academic Press, London, UK (2002)
6. Chiou A, Chen LH, Chen SK. Foodborne illness in Taiwan 1981-1989. Food Aust. 43: 70-71 (1991)
7. Lee WC, Sakai T, Lee MJ, Hamakawa M, Lee SM, Lee IM. An epidemiological study of food poisoning in Korea and Japan. Int. J. Food Microbiol. 29: 141-148 (1996)
8. Wong HC, Liu SH, Ku W, Lee IY, Wang TK, Lee YS, Lee CL, Kuo LP, Shih DYC. Characterization of *Vibrio parahaemolyticus* isolates obtained from foodborne illness outbreaks during 1992 through 1995 in Taiwan. J. Food Protect. 63: 900-906 (2000)
9. Lee WC, Lee MJ, Kim JS, Park SY. Foodborne illness outbreaks in Korea and Japan studied retrospectively. J. Food Protect. 64: 899-902 (2001)

10. Park MY. Urease-positive *Vibrio parahaemolyticus* isolated from Kamak Bay in Yeosu in 2002 and 2003. Food Sci. Biotechnol. 13: 508-511 (2004)
11. KFDA (Korea Food and Drug Administration), Annual Report of Foodborne Outbreak. Available from: http://www.kfda.go.kr/main/html/service1.htm. Accessed Mar. 5, 2006.
12. Chan K, Woo ML, Lam LY, French GL. *Vibrio parahaemolyticus* and other halophilic Vibrios associated with seafood in Hong Kong. J. Appl. Bacteriol. 66: 57-64 (1989)
13. Elliot EL, Kaysner CA, Jackson L, Tamplin ML. *Vibrio cholerae, V. parahaemolyticus, V. vulnificus,* and other *Vibrio* spp. pp. 111-140. In: FDA Bacteriological Analytical Manual. 7th ed. Association of Official Analytical Chemists International, Arlington, VA, USA (1992)
14. DePaola A, Hopkins LH, McPhearson RM. Evaluation of four methods for enumeration of *Vibrio parahaemolyticus*. Appl. Environ. Microb. 54: 617-618 (1988)
15. Blodget RJ, Garthright WE. Several MPN models for serial dilutions with suppressed growth at low dilutions. Food Microbiol. 15: 91-99 (1998)
16. Pang SJ, Xiao T, Bao Y. Dynamic changes of total bacteria and *Vibrio* in an integrated seaweed-abalone culture system. Aquaculture 252: 289-297 (2006)
17. Chiang ML, Ho WL, Chou CC. Response of *Vibrio parahaemolyticus* to ethanol shock. Food Microbiol. 23: 461-467 (2006)
18. Huq A, West PA, Amall EB, Huq MI, Colwell RR. Influence of water temperature, salinity, and pH on survival and growth of toxigenic *Vibrio* cholerae Serovar O1 associated with live Copepods in laboratory microcosms. Appl. Environ. Microb. 48: 420-424. (1984)
19. Balebona MC, Moriñigo MA, Faris A, Krovacek K, Mânsson L, Bordas MA, Borrego JJ. Influence of salinity and pH on the adhsion of pathogenic *Vibrio* strains to *Sparus aurata* skin mucus. Aquaculture 132: 113-120 (1995)
20. Prayitno SB, Latchford JW. Experimental infections of crustaceans with luminous bacteria related to *Photobacterium* and *Vibrio*. Effects of salinity and pH on infectiosity. Aquaculture 132: 105-112 (1995)
21. Ama AA, Hamdy MK, Toledo RT. Effects of heating, pH, and thermoradiation on inactivation of *Vibrio vulnificus*. Food Microbiol. 11: 215-227 (1994)
22. Hijarrubia MJ, Lázaro B, Suñén E, Fernándeztorga A. Survival of *Vibrio vulnificus* under pH, salinity, and temperature combined stress. Food Microbiol. 13: 193-199 (1996)
23. Azanza PV, Buckel KA, Fleet GH. Effect of diluents on the enumeration of *Vibrio vulnificus*. Int. J. Food Microbiol. 30: 385-390 (1996)
24. Miles DW, Ross T, Olley J, McMeekin TA. Development and evaluation of a predictive model for the effect of temperature and water activity on the growth rate of *Vibrio parahaemolyticus*. Int. J. Food Microbiol. 38: 133-142 (1997)
25. Bej AK, Patterson DP, Brasher CWL, Vickery MC, Jones DD, Kaysner C. Detection of total and hemolysin-porducing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl, tdh,* and *trh*. J. Microbiol. Meth. 36: 215-225 (1999)
26. Kim SM, Noh BS, Kim HY, Park SJ, Ji GE. Analysis of *Vibrio parahaemolyticus* OMPs and production of antibodies against OMPs. Food Sci. Biotechnol. 14: 410-412 (2005)
27. Korea Oceanographic Data Center, Searched at the data of salinity and seawater temperature of the coast of Korea in 2004. Available from: http://kodc2.nfrdi.re.kr:8001/home/kor/main/index.php. Accessed Apr. 2, 2005.
28. Steel RGD, Torrie JH. Principles and Procedure Statistics, with Special Reference to the Biological Sciences. McGraw-Hill, New York, NY, USA. pp. 107-109 (1960)
29. O'Mahony M. Sensory Evaluation of Food, Statistical Methods and Procedures. Marcel Dekker, Inc., New York, NY, USA. pp. 153-170 (1986)
30. Lee KK, Liu PC, Huang CY. *Vibrio parahaemolyticus* infectious for both humans and edible mollusk abalone. Microbes Infect. 5: 481-485 (2003)
31. Ogden ID, Brown GC, Gallacher S. Garthwaites PH, Gennari M, Gonzalez MP, Jørgensen LB, Lunestad BT, MacRae M, Nunes MC, Petersen AC, Rosnes JT, Vliegenthart J. An interlaboratory study to find an alternative to the MPN technique for enumerating *Escherichia coli* in shellfish. Int. J. Food Microbiol. 40: 57-64 (1998)
32. Liew WS, Leisner JJ, Rusul G, Radu S, Rassip A. Survival of *Vibrio* spp. including inoculated *V. cholera* 0139 during heat-treatment of cockles (*Anadara granosa*). Int. J. Food Microbiol. 42: 167-173 (1998)