VERSITA

Biologia **68**/6: 1017—1021, 2013
Section Cellular and Molecular Biology
DOI: 10.2478/s11756-013-0251-x

Use of formic acid to control vibriosis in shrimp aquaculture*

Derek Adams & Raj Boopathy**

Department of Biological Sciences, Nicholls State University, Thibodaux, LA 70310 USA;
e-mail: Ramaraj.Boopathy@nicholls.edu

Abstract: Luminous vibriosis is a shrimp disease that causes major economic losses in shrimp industry as a result of massive shrimp kills due to bacterial infection caused by *Vibrio* species. Use of antibiotics to control *Vibrio* in shrimp aquaculture is not allowed in the United States and so it is necessary to develop an alternative pathogen control method for shrimp production. Short-chain fatty acids have been used as food preservatives for a long time. Organic acids are commonly added in feeds in animal production, such as chicken, pig, and cattle. In this study, growth inhibition effects of formic acid on five selected *Vibrio* species, namely *Vibrio alginolyticus*, *Vibrio cholerae*, *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* were studied. The *Vibrio* bacteria were grown on both solid and liquid media using Muller-Hinton agar and alkaline peptone water, respectively, with various concentrations of formic acid. Bacterial growth was monitored in the liquid media using optical density method. The results showed significant inhibition of growth of all five *Vibrio* species by formic acid at low concentration. The effective concentration (EC_{50}) values were calculated for all five *Vibrio* species, which were less than 0.039% of formic acid. The results are encouraging to supplement formic acid in the shrimp feed as a control mechanism to reduce *Vibrio* outbreak in shrimp aquaculture system.

Key words: vibriosis; luminescence; EC_{50}; shrimp aquaculture; antibiotic resistance.

Abbreviations: APW, alkaline peptone water; EC_{50}, effective concentration 50; MHA, Muller-Hinton agar.

Introduction

Global aquaculture production has been growing rapidly since the 1950s. In 1982, shrimp farming accounted for only 5% of the world shrimp production achieving 25% in 1990 (Gillet 2008). Currently more than 40% of the global shrimp production come from aquaculture.

The major bacterial diseases in shrimp aquaculture include vibriosis caused by *Vibrio* species and necrotizing hepatopancreatitis caused by obligate intracellular rickettsia-like bacteria (Lightner 2005). Vibriosis is an infectious disease caused by *Vibrio* species (Lightner 1996). Within *Vibrio* species, *Vibrio harveyi*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, and *Vibrio alginolyticus* are most frequently reported in shrimp hatchery, nursery and grow out ponds (Lightner 1996). Vibriosis in shrimp ponds occurs abruptly, and infection spreads rapidly for several days to two weeks (Brock & LeaMaster 1992). As a result, high mortality sometimes with luminescence in shrimp ponds occurs (Lightner 1996). Luminescence, cloudiness of shrimp musculature, red discoloration of appendages, and weak swimming at the pond surfaces or edges are the visible signs of infection in penaeid shrimp (Brock & LeaMaster 1992). The large numbers of bacteria are assumed to exist in the infected shrimp, so isolation of organisms from tissue and haemolymph is performed to confirm the diagnosis (Lightner 1996). *Vibrio* species are ubiquitous in the marine and estuarine environment (Krieg et al. 1984), thus the routes of vibrio infection are presumed to be through the pit, including puncture wounds, on the exoskeleton and through oral ingestion (Lightner & McVey 1993).

Antibiotic resistance of bacteria impacts the choice of therapeutic drugs humans and animals can use. Antibiotics are often used in some shrimp farms in the world except in USA where the use of antibiotic in shrimp aquaculture is banned (Graslund & Bengtsson 2001; Le & Munekage 2004; Lyle-Fritch et al. 2006). Not only emergences of antibiotic-resistant bacteria but also antibiotic residues in food-producing animals pose health concerns in humans and animals. In order to control infectious diseases in shrimp aquaculture, reduction in the use of antibiotics accompanied with various strategies, such as improvement of both pond water quality and the host health, are recommended (Hernandez Serrano 2005).

There are several alternative strategies to control vibriosis, which include use of probiotic bacteria in

* Based on a contribution presented at the *International Conference on Industrial Biotechnology* (ICIB-2012), November 21–23, 2012, Punjabi University, Patiala (Pb.), India
** Corresponding author

shrimp ponds. Probiotics are basically live bacterial additives that bring beneficial health effects to the host animals (Verschuere et al. 2000). Short-chain fatty acids include formic, acetic, propionic, and butyric acid. The generally known antibacterial property of these organic acids is acidification of the cytoplasm (Doyle et al. 2001). Some organic acids are the natural metabolic products of organisms, and they have been used as food preservatives for a long time. Organic acids are also used as feed additives to control pathogens in animal husbandry (Iba & Berchieri 1995; Franco et al. 2004). Several studies have proven that inclusion of organic acids in diets suppresses pathogenic bacterial growth in gastrointestinal tracts of poultry and swine (Iba & Berchieri 1995; Franco et al. 2004). Other studies showed both short-chain and medium-chain fatty acids are effective bactericides of pathogenic *Salmonella* and *Campylobacter* species (Khan & Katamay 1968; Chaveerach et al. 2002). In shrimp aquaculture, organic acids are not used to control bacterial infections, and the effects of organic acids in shrimp aquaculture have not been studied in detail (Saori & Boopathy 2011). As seen in human food preservatives and terrestrial animal feeds, the use of organic acids has a potential to become alternatives to antibiotics in aquaculture. In our previous study, we tested variety of organic acids on *V. harveyi* and found formic acid as a potential inhibitor of its growth (Mine & Boopathy 2011). We extend the current study with a major objective of finding the effect of formic acid on various species of *Vibrio*, including *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus,* and *V. cholerae*.

Material and methods

Organism and chemicals

Vibrio harveyi (ATCC 14126), *V. parahaemolyticus,*(ATCC 17802), *V. vulnificus* (ATCC 29307), *V. alginolyticus* (ATCC 17750), and *V. cholerae* (ATCC 14035) were obtained from the American Type Culture Collection, Manassas, VA, USA. All *Vibrio* bacteria were maintained in alkaline peptone water (APW) supplemented with 2% NaCl, adjusted to pH 8.4. The cultures were maintained at room temperature (19–24 ℃) and were sub-cultured weekly. *V. harveyi* used in this study is known to be pathogenic to shrimp (Lightner 1996). All chemicals including formic acid, acetic acid, butyric acid, and propionic acid, reagents, and media were obtained from Fisher Scientific (St. Louis, MO, USA).

Disc diffusion assay

The organic acids, formic acid, acetic acid, propionic acid, and butyric acid, were used to make 5, 10, 25, 50, and 100% organic acid solutions by diluting with de-ionized water.

The method used to inoculate bacteria on agar was based on the Centers for Disease Control and Prevention's method for antimicrobial susceptibility testing of *V. cholerae* (Anonymous 1999). Muller-Hinton agar (MHA) supplemented with 2% NaCl and adjusted pH to 8.4 was used for disc diffusion assays run in triplicate. Twenty-five ml of MHA was poured to 100 mL Petri plates to a 4 mm depth. Inocula were prepared with one loopful of stock culture into 10 mL APW (2% NaCl, pH 8.4) followed by overnight incubation at 26 ℃. Prior to streaking inocula onto MHA, turbidity of the bacterial suspension in APW was adjusted to absorbance of 0.08-0.1 at 600 nm wavelength by adding APW. Each culture was streaked over the entire MHA surface three times with sterile cotton swabs. Within 10 minutes after streaking, a filter paper (Whatman) disc (diameter, 7.03 mm) was placed on the agar surface, and it was saturated with 2–3 µL of an organic acid solution using a micropipette. Petri plates were inverted, and incubated at 26 ℃ for 16–18 hours. After incubation, the diameter of the zones of complete inhibition was measured and recorded in millimeters.

Determination of effective concentration (EC_{50}) of organic acids

Based on the results from the disc diffusion assay formic acid was chosen for further study. Formic acid solutions with 0.025, 0.03, 0.035, 0.04, 0.045, and 0.05% were prepared by diluting with APW (2% NaCl, pH 8.4). Fifty mL of various concentrations of formic acid solutions were dispensed into 100 mL culture bottles in triplicate with ingredients of APW medium. The control contained no formic acid. Two hundreds µL of overnight culture of *Vibrio* adjusted to 0.08 absorbance at 600 nm wavelength was inoculated to each culture bottle. Bacterial growth was monitored by absorbance using a Genesys 20 Visible Spectrophotometer (Thermo Fisher Scientific, Inc.) at the wavelength of 600 nm, and pH was measured with a calibrated UltraBASiC pH/mV meter (Denver Instruments). Both absorbance and pH were measured for 14 days, at every 12 hours for the first 4 days and every 24 hours for the rest of 10 days.

Statistical analysis

The zone of inhibition was subjected to an analysis of variance (ANOVA) test ($p \leq 0.05$) followed by a Tukey's *post hoc* analysis (SAS/Genetics Version 9.1.3, 2003, SAS Institute, Cary, NC, USA).

Results and discussion

Relative toxicity of organic acids

The relative toxicity of formic acid, acetic acid, propionic acid, and butyric acid on various shrimp species was studied using a filter paper disc assay. Sterile filter paper discs were saturated with various concentrations of organic acids and placed over the lawn of *Vibrio* plate, as described above. The results indicated that formic acid inhibited bacterial growth with the biggest zone of inhibition in all concentrations tested compared to acetic, propionic, and butyric acids. Analysis of variance followed by Tukey's test was used to compare the effects of these acids. Formic acid was the most effective followed by acetic acid, propionic acid, and butyric acid (data not shown). As a result of this screen, further experiment was conducted only with formic acid.

Determination of effective concentration (EC_{50}) of formic acid for various Vibrio species

An EC_{50} assay was done on various *Vibrio* species with formic acid at different concentrations. Bacterial growth was monitored using optical density measuring the absorbance at 600 nm wavelength. The concentration of formic acid used was 0.025, 0.03, 0.035, 0.04, 0.045, and 0.5%. A control was used without any formic

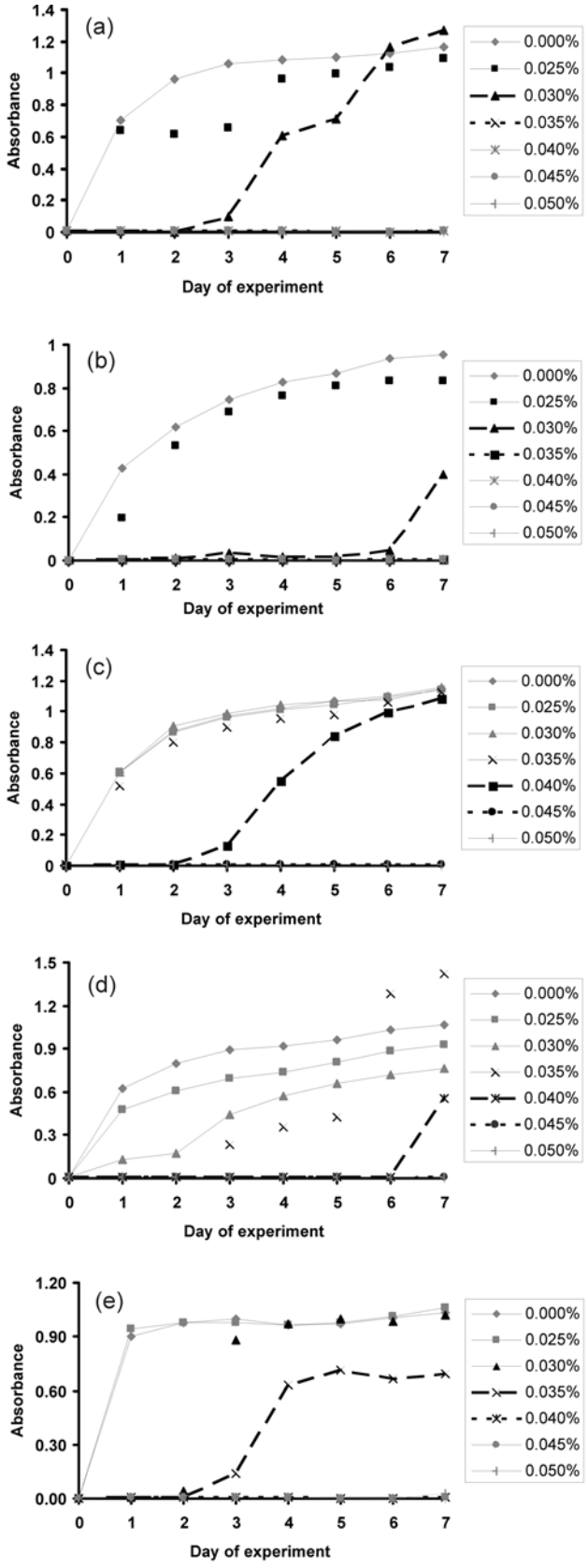

Fig. 1. Mean absorbance (as a representation of bacterial growth) for *Vibrio alginolyticus* (a), *Vibrio harveyi* (b), *Vibrio cholerae* (c), *Vibrio vulnificus* (d) and *Vibrio parahaemolyticus* (e) exposed to various levels of formic acid. The solid black line represents the level lower than the level that provided a noticeable effect (large dashed line) and the small dashed line represents the lowest level of formic acid that provided full inhibition for the duration of the experiment.

acid. Bacterial growth curve for the individual *Vibrio* bacteria is presented in Figure 1. The results indicated that formic acid at the concentration of 0.025% did not have any effect on all *Vibrio* species tested. However, 0.03% formic acid showed inhibition at the beginning of the experiment up to five days of incubation and later growth was observed indicating a lag period. The concentrations of 0.035% and higher completely inhibited *V. harveyi* growth (Fig. 1b). Maximum growth was observed in control without formic acid. Similarly all other *Vibrio* bacteria showed no inhibitory effect at the concentration of 0.025% formic acid, however, the lag period varied among species for concentrations >0.025%. The concentration of 0.05% formic acid completely inhibited all *Vibrio* species.

The pH of the culture media for *V. harveyi* and other *Vibrio* species were monitored. In the low concentrations of formic acid, such as 0.025 and 0.03%, the initial drop in pH was increased to above neutral pH by *V. harveyi*. This pH recovery might be due to solute production by the bacteria, which explains better growth in these two concentrations. However, the pH recovery was lost when the formic acid concentration was increased to 0.035% and higher. Similar trend was observed in all other *Vibrio* species (data not shown).

An EC_{50} plot was constructed by taking the growth reading at 96 hour (maximum growth in control) for all formic acid concentrations for *V. harveyi* as described earlier (Mine & Boopathy 2011). The growth was observed in control and the drop in bacterial growth was directly proportional to formic acid concentration in the media. The 96 hour EC_{50} value for formic acid was 0.023%. Formic acid at the concentration of 0.035% totally inhibited *V. harveyi* growth (data not shown). The EC_{50} values for all other *Vibrio* species are given in Table 1. This result indicates that formic acid at the concentration of 0.035% could be used in an aquaculture farm to control *Vibrio* infection either as a disinfectant or as pre-biotic supplement with the shrimp feed.

Aquaculture is a rapidly growing industry in agriculture with its protein production catching up with that of terrestrial animal production (O'Bryen & Lee 2003). Shrimp is a valued seafood worldwide, and investments from various international services and shrimp-importing countries are made in shrimp aquaculture development. However, vibriosis in shrimp farms causes major losses of investments. Disease prevention by screening for pathogenic microorganisms, as in those found in the specific pathogen list, is not very practical for common marine fauna such as *V. harveyi*. Besides the virulence of *V. harveyi*, the health of hosts, which may be predetermined by environmental conditions and life stages, affects susceptibility to *Vibrio* infections. Specially, farms in developing countries, which use coastal areas as farm grounds might be more influenced by environmental changes, compared to indoor farms that use raceway systems. Thus, complete prevention of vibriosis seems difficult. Responding to the early signs of vibriosis is important, and a sustainable

Table 1. EC_{50} values for all *Vibrio* species.

Organism	Observed maximum absorbance	Half maximum	EC_{50}
Vibrio harveyi	0.825	0.4125	0.023%
Vibrio alginolyticus	1.085	0.5425	0.026%
Vibrio parahemalyticus	0.997	0.4985	0.030%
Vibrio vulnificus	0.918	0.459	0.028%
Vibrio cholerae	1.016	0.508	0.039%

way to do so is needed. As a potential alternative to antibiotics, this study focused on the effect of formic acid on the shrimp pathogen, *V. harveyi*.

This study showed the minimal inhibition concentrations and EC_{50} values of formic acid for five *Vibrio* species. The relative toxicity of the organic acids coincides with the relative lipophilicity. The estimated 96 hour EC_{50} value was 0.023% formic acid for five different *Vibrio* species. These growth patterns agree with the pH change in the media. The pH value of the control remained neutral to slightly alkaline. The pH value in the completely inhibited treatment showed $\leq$5, and treatments with an initial pH of above 5 showed a later pH increase to neutral. These observations indicate that it is more likely that *V. harveyi* and other *Vibrio* species growth is inhibited when the media pH is below or at 5. When the media pH is above 5, *V. harveyi* is able to adapt to the acidic environment and survive. It is not known if the growth inhibitory effect is solely due to the pH value of below 5. If the inhibitory effect is due to the amount of undissociated form at the pH below 5, the relative amount of undissociated forms of organic acids should be able to explain the relative toxicity. The amount of undissociated forms of organic acids at a certain pH can be calculated with the Henderson-Hasselbalch equation (Saarikoski & Vilukseta 1981). The calculated proportion of undissociated forms at pH 5 of four organic acids is as follows: 5.32% formic acid, 35.46% acetic acid, 42.57% propionic acid, and 39.78% butyric acid. According to this calculation, at pH 5, undissociated forms of formic acid is the least among four organic acids, and in acetic, propionic, and butyric acid solutions at pH 5, relatively the same proportion of undissociated forms exist, because of the relatively close pK_a values of the three acids. This indicates that formic acid has a different antimicrobial action mode other than intracellular acidification by undissociated forms of organic acids. The study of a human gastrointestinal pathogen *V. parahaemolyticus* (Tanaka et al. 2008) showed acid adaptation of *V. parahaemolyticus* with an accumulation of the decarboxylation product of lysine. Lysine decarboxylation is the mechanism thought to be related to both decrease in the intracellular proton concentration and excretion of the basic molecule amine as an acid tolerance response. As seen in the recovery of pH from acidic to neutral in this study, *V. harveyi* and other *Vibrio* species may have a similar mechanism to acid stress. This might additionally explain the strong inhibitory effect of formic acid. Formic acid is known to inhibit decarboxylases even at pH values in which substantial amounts of dissociated forms exist (Lueck 1980). According to Krieg et al (1984), *Vibrio* species can utilize C_2-C_{10} monocarboxylic fatty acids as a carbon source. This also supports that formic acid, or C_1 monocarboxylic fatty acid, is less favourable for vibrio growth. The potential use of formic acid in shrimp aquaculture either as a disinfectant or as a diet supplement in shrimp feed should be explored further to control vibriosis in shrimp aquaculture farms. This study was the extension of our previous work on the effect of organic acids on one species of vibrio bacteria, namely, *V. harveyi* (Mine & Boopathy 2011). Here we demonstrate the effect of formic acid on multiple species of *Vibrio*, which are a major threat to shrimp aquaculture industry.

Conclusions

The organic acids are the natural metabolic products of organisms, and many are used for human food preservatives for their antimicrobial properties. In the mature agricultural sectors, such as swine and poultry industries, the organic acids are used as food additives to control pathogens. The practice of using organic acid, such as formic acid in shrimp aquaculture, is not yet well established. This study showed the use of formic acid for effective pathogen control against *Vibrio* species. The growth of *V. harveyi* and other *Vibrio* species was inhibited by formic acid at low concentration. The minimum inhibition concentration of formic acid on *V. harveyi* and the EC_{50} at 96 hour incubation were determined to be 0.035% and 0.023%, respectively.

Acknowledgements

This work was funded by a grant from the US Department of Agriculture's National Institute of Food and Agriculture (NIFA) to United States Marine Shrimp Farming Program (USMSFP).

References

Anonymous. 1999. Laboratory Methods for the Diagnosis of Epidemic Dysentery and Cholera, pp. 62-72. Centers for Disease Control and Prevention. Atlanta, Georgia, USA.

Brock J.A. & LeaMaster B. 1992. A look at the principal bacterial, fungal and parasitic diseases of farmed shrimp, pp. 212-226. In: Wyban J. (ed.) Proceedings of the Special Session on Shrimp Farming. World Aquaculture Society, Baton Rouge, LA, USA.

Chaveerach P., Keuzenkamp D.A., Urlings H.A.P., Lipman L.J.A. & van Knapen F. 2002. *In vitro* study on the effect

of organic acids on *Campylobacter jenuni/coli* populations in mixtures of water and feed. Poultry Sci. **81:** 621-628.

Doyle M.P., Beuchat L.R. & Montville T.J. 2001. Food Microbiology: Fundamentals and Frontiers. American Society for Microbiology, Washington, D.C., 872 pp.

Franco L.D., Fondevila M., Lobera M.B. & Castrillo C. 2005. Effect of combinations of organic acids in weaned pig diets on microbial species of digestive tract contents and their response on digestibility. J. Anim. Physiol. Anim. Nutr. **89:** 88-93.

Gillett R. 2008. Global Study of Shrimp Fisheries. FAO Fisheries Technical Paper, No. 475. Rome, FAO, 331 pp.

Graslund S & Bengtsson B.K. 2001. Chemicals and biological products used in south-east Asian shrimp farming, and their potential impact on the environment – a review. Sci. Total Environ. **280:** 93-131.

Hernandez Serrano P. 2005. Responsible Use of Antibiotics in Aquaculture. FAO Fisheries Technical Paper, No. 469. Rome, FAO, 97 pp.

Iba A.M. & Berchieri A. 1995. Studies on the use of a formic acid-propionic acid mixture (Bio-add^{TM}) to control experimental *Salmonella* infection in broiler chickens. Avian Pathol. **24:** 303-311.

Khan M. & Katamay M. 1969. Antagonistic effect of fatty acids against *Salmonella* in meat and bone meal. Appl. Microbiol. **17:** 402-404.

Krieg N.R., Holt J.G., Murray R.G.E., Brenner D.J., Bryant M.P., Moulder J.W., Pfennig N., Sneath P.H.A. & Staley J.T. 1984. In: Bergey's Manual of Systematic Bacteriology, Vol. 1, pp. 518-519. Williams & Wilkins, Baltimore, MD, USA.

Le T.X. & Munekage Y. 2004. Residues of selected antibiotics in water and mud from shrimp ponds in mangrove areas in Vietnam. Mar. Pollut. Bull. **49:** 922-929.

Lightner D.V. 1996. In: A Handbook of Shrimp Pathology and Diagnostic Procedures for Disease of Cultures Penaeid Shrimp, pp. 126-145. World Aquaculture Society, Baton Rouge, LA, USA.

Lightner D.V. 2005. Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance. J. World Aquacult. Soc. **36:** 229-248.

Lightner D.V. & McVey J.P. 1993. CRC Handbook of Mariculture, Vol. 1: Crustacean Aquaculture. CRC Press, Boca Raton, FL, USA, 420 pp.

Lueck E. 1980. Antimicrobial Food Additives. Spriger-Verlag, Berlin, Heidelberg, New York, 162 pp.

Lyle-Fritch L.P., Romero-Beltran E. & Paez-Osuna F. 2006. A survey on use of the chemical and biological products for shrimp farming in Sinaloa, NW Mexico. Aquacult. Eng. **35:** 135-146.

O'Bryen P.J. & Lee C. 2003. Discussion summary on biosecurity in aquaculture production systems: exclusion of pathogens and other undesirables, pp. 275-293. The World Aquaculture Society, Baton Rouge, LA USA.

Saarikoski J. & Vilukseta M. 1981. Influence of pH on the toxicity of substituted phenols to fish. Arch. Environ. Contam. Toxicol. **10:** 747-753.

Saori M. & Boopathy R. 2011. Effect of organic acids on shrimp pathogen, *Vibrio harveyi*. Curr. Microbiol. **63:** 1-7.

Tanaka Y., Kimura B., Takahashi H., Watanabe T., Obata H., Kai A., Morozumi S. & Fuji T. 2008. Lysine decarboxylase of *Vibrio parahaemolyticus*: kinetics of transcription and role in acid resistance. J. Appl. Microbiol. **104:** 1283-1293.

Verschuere L., Rombaut G., Sorgeloos P. & Verstraete W. 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. Rev. **64:** 655-671.

Received November 3, 2012
Accepted February 26, 2013