



# Current Research in Bacteriology

ISSN 1994-5426

**science**  
alert

**ANSI***net*  
an open access publisher  
<http://ansinet.com>

## Anti-*Vibrio harveyi* Property of *Micrococcus luteus* Isolated from Rearing Water under Biofloc Technology Culture System

<sup>1,2</sup>April Rose B. Barcenal, <sup>1</sup>Rex Ferdinand M. Traifalgar and <sup>1</sup>Valeriano L. Corre Jr

<sup>1</sup>University of the Philippines Visayas, College of Fisheries and Ocean Sciences, Institute of Aquaculture, Miagao, Iloilo, Philippines

<sup>2</sup>University of Antique Tario-Lim Memorial Campus, College of Fisheries, Tibiao, Antique

Corresponding Author: Rex Ferdinand M. Traifalgar, University of the Philippines Visayas, College of Fisheries and Ocean Sciences, Institute of Aquaculture, Miagao, Iloilo, Philippines

### ABSTRACT

Biofloc culture system of shrimp is currently gaining wide acceptance since it increases production efficiency and lowers the risk of disease occurrence. The biofloc culture system is known to improve the water quality and it has been observed that this culture system tends to inhibit the population of pathogenic *Vibrios*. It is the intention of present work to isolate, characterize and identify bacterial species with potent anti-*Vibrio* activity from the biofloc technology culture system. Results indicate the presence of bacteria with Vibriostatic activity in the biofloc culture system. Identification revealed that the active bacteria is *Micrococcus luteus* exhibiting an optimum *Vibrio* lytic activity at 20 ppt and at a pH of around 8. Competitive gut colonization assay shows that the isolated bacteria has a higher gut colonizing activity than that of the pathogenic *Vibrio harveyi*. In conclusion *M. luteus* isolated from the biofloc technology culture system exhibited *Vibrio* lytic activity *in vitro* and gut colonizing activity *in vivo*.

**Key words:** Biofloc technology, antagonistic property, *Vibrio harveyi*, *Micrococcus luteus*

### INTRODUCTION

Shrimp is known as one of the most important internationally traded fisheries commodity in terms of value. In many tropical developing countries, it is the most valuable fisheries export and a significant source of employment (FAO., 2009). The intensification of the culture practice as a response to the market demand has led to massive problems which included the spread of diseases. Among the diseases, luminous vibriosis caused specifically by *Vibrio harveyi* (FAO, 2014) is known as the most important bacterial pathogen in cultured shrimps and reported to cause mortalities of up to 100% in hatcheries and grow-out ponds (Karunasagar *et al.*, 1994). *V. harveyi* could also be considered as a primary causative agent of diseases that can lead to secondary viral infection such as White Spot Syndrome Virus (WSSV) (De la Peña *et al.*, 2003).

Biofloc Culture Technology (BFT) is a new innovation in aquaculture that allows super intensive culture of shrimp with minimal water exchange (Avnimelech, 2007). The technology was basically derived from the concepts of waste water treatment that involves the addition of carbon sources to promote bacterial assimilation of nitrogenous organic wastes. Addition of carbon hastens bacterial metabolism leading to the sequestration and utilization of dissolve nitrogen for protein synthesis and to fuel rapid cellular proliferation that eventually results to the formation of visible bacterial biomass termed as biofloc (Crab *et al.*, 2007). The Biofloc culture system has been also

documented to lower the risk of disease occurrence (Hargreaves, 2013). Although, the favorable conditions in the BFT system is mainly driven by bacterial communities, no known negative effects in the cultured organism by these bacteria were reported. Studies suggested that heterotrophic bacteria in the system could have probiotic-like effects (Kesarcodi-Watson *et al.*, 2008). Wasielesky *et al.* (2006) and Ballester *et al.* (2010) suggested that BFT can improve shrimp health by preventing allochthonous pathogenic organisms from invading the system through competitive exclusion. However, isolation of specific bacteria with anti-bacterial property specifically *V. harveyi* from shrimp culture under BFT rearing water has not been reported to date. This study aimed to isolate and identify bacteria with anti-*V. harveyi* property from BFT culture system.

## MATERIALS AND METHODS

**Bacterial isolation and purification:** Water sample used for the isolation was obtained from the Biofloc rearing ponds of *Litopenaeus vannamei* of the University of the Philippines Visayas (UPV)-Department of Science and Technology (DOST) Shrimp Project 2 in Miag, ao, Iloilo, Philippines. Floc reading was ensured to be in stage five corresponding to about 15 mL Total Settleable Solids (TSS) (measured using Imhoff cone) during the isolation. This is to assure that the system is at its peak of bacterial abundance. The isolation method was based on the protocol described by Gullian *et al.* (2004) with modifications. Water samples were collected using a sterile container, serially diluted and  $10^{-3}$  and  $10^{-4}$  dilutions were plated out in Tryptic Soy Agar (TSA) by spread plate. After 24 h of incubation, unique colonies were enumerated and purified. Stock cultures were also made in slant 1/3 strength Luria Bertani (Lb) agar with 2% NaCl and were stored in the refrigerator until further use.

***In vitro* antagonistic assays:** To isolate bacteria with anti-*Vibrio* activity, selection antagonistic assay was conducted using Spot-on-the-Lawn method described by Cadirci and Citak (2005) with some modifications. Three hundred microliter of 24 h culture *V. harveyi* (PN-9801) obtained from Southeast Asia Fisheries Development Center-Aquaculture Department (SEAFDEC-AQD) was spread in 1/3 strength Lb agar (2% NaCl) and surface of the agar was allowed to dry. Twenty-four hour culture of each of the isolated bacteria was then spotted in the surface of the media previously inoculated with *V. harveyi* and incubated at room temperature for 24 h. Bacterial isolate exhibiting zones of inhibition in *V. harveyi* lawn were isolated and characterized by the Gram-staining method.

Bacteria with antagonistic property against live *V. harveyi* were then checked for their lytic ability on heat-killed *V. harveyi* cells. The experiment was conducted using a modified Spot-on-lawn (Cadirci and Citak, 2005) and Double Layer Method (Than *et al.*, 2004). Heat-killed cells of *V. harveyi* were prepared and incorporated in 1/3 strength Lb agar with varying NaCl content and pH conditions. The NaCl inclusions that were used were 0, 0.5, 1, 2 and 3% while the pH varied from 6, 7 and 8. Twenty four hour culture of the test bacterial isolate were then spotted in the surface of the media and incubated for 24 h. Cellular lytic zones were observed and measured.

**Bacterial identification:** Identification was done using Gram staining method and using Biolog 10 BBL Crystal™ Identification Systems Id Kit. Four to five pure colonies of 24 h cultured bacteria in TSA plates were suspended in BBL Crystal™ ANR, GP, RGP, N/H ID Inoculum Fluid, vortexed and used to fill 30 reaction wells of the BBL Crystal™ GP ID panel lids. The lids contained 29 dehydrated substrates and a fluorescence control on tips of plastic prongs. After 18-24 h

incubation at room temperature, the wells were examined for direct color changes or presence of fluorescence with the aid of UV Transilluminator that resulted from metabolic activities of the microorganisms. Positive or negative results were determined based on the color manual guide. The resulting pattern of the 29 reactions was converted into a ten-digit profile number that was used as the basis for identification using the BBL Crystal™ GP ID Data base.

***In vivo* antagonistic property assay:** The experiment was conducted based on the methods of Gullian *et al.* (2004) and Sivakumar *et al.* (2012) with modifications. A Complete Randomized Design (CRD) was employed with three experimental treatments run in triplicate. In the first treatment (T1) group of shrimp were cultured without the addition of the test isolate of *V. harveyi*. Treatment 2 (T2) consists of the shrimp group receiving daily inoculum of the test isolates for 3 days and *V. harveyi* was added on the fourth day. The third treatment (T3) is a group of shrimp that received only *V. harveyi* on the fourth day of the culture.

The experiment was conducted using tanks with 15 L of filtered and ultraviolet-treated 20 ppt water that were stocked with fifteen  $1.23 \pm 0.07$  g shrimp. Bacterial inoculum with  $10^5$  bacteria  $\text{mL}^{-1}$  concentration was inoculated in tanks of Treatment 2 for three successive days and water exchange was observed 20 h after each inoculation. On the fourth day, inoculum of *V. harveyi* was added at a concentration of  $10^5$  bacteria  $\text{mL}^{-1}$  in Treatments 2 and 3. The control treatment did not receive any inoculum addition. Hepatopancreas of 5 shrimp from each tank of all the treatments were extracted 24 h after the addition of *V. harveyi*. Seven serial dilutions (1/10) were prepared and  $10^{-4}$  to  $10^{-7}$  dilutions were plated out on TSA (2% NaCl) and TCBS agar (2% NaCl). Total *Vibrio harveyi* count from the hepatopancreas was enumerated on TCBS agar after 24 h of incubation at room temperature. Antagonistic property was determined by comparing the total number of *V. harveyi* present in the hepatopancreas of shrimp exposed to the experimental treatments.

**Statistical analysis:** A one-way analysis of variance (ANOVA) was used to determine the significance of differences in means of the gathered data. Results with significant differences were subjected to post hoc analysis using Tukey's test. SPSS16 was used to analyze the results of the experiment.

## RESULTS

Among the isolates, one colony showed strong antagonism against *V. harveyi* as indicated by the inhibition zone of  $16.66 \pm 2.33$  mm (Fig. 1). The colony was observed to have circular form, entire margin, convex elevation and opaque yellow coloration. Gram staining indicated that the isolated bacteria is gram positive and was identified as *Micrococcus luteus* by the Biolog 10 BBL Crystal™ Identification Systems Id Kit. It showed positive response in 4 MU- $\beta$ -D-glucoside, L-valine-AMC, L-phenylalanine, L-pyroglutamic acid-AMC, L-tryptophan, 4 MU-N-acetyl- $\beta$ -D-glucosaminide, 4 MU-phosphate, Proline and Leucine-p-nitroanilide, p-n-p-Phosphate, Urea Arginine (Table 1).

The isolated bacteria exhibited a strong lytic ability on *V. harveyi* cells. At 0,5,10,20 and 30 ppt the observed utilization zone were  $11 \pm 0.1$  mm,  $14.50 \pm 0.50$ ,  $14.50 \pm 0.50$ ,  $16.00 \pm 1.50$  and  $10.00 \pm 1.00$  mm, respectively (Fig. 2). The lytic activity was highest at 20 ppt but has no significant difference compared to the utilization zones observed from other salinities. At pH 6,7 and 8 levels, the utilization zones of the isolate were  $12.50 \pm 1.50$ ,  $17.00 \pm 10$  and  $14.00 \pm 2.00$  mm, respectively (Fig. 3). The activity was highest at pH 7 but was not found to be significant from pH 6 and 8.

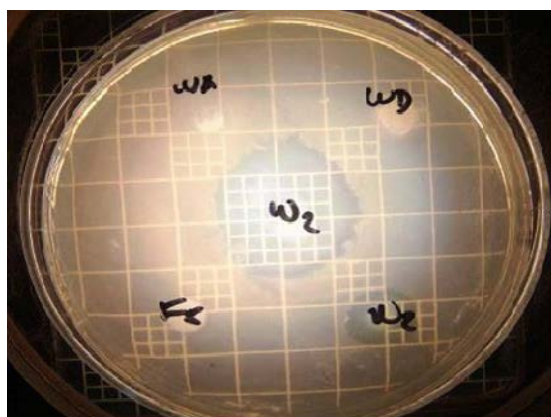


Fig. 1: Spot-on-the-lawn result of the different isolates from the biofloc set-up rearing water after 24 h of incubation at room temperature in Lb agar (2% NaCl) with only isolate W2 (identified as *Micrococcus luteus*) showing inhibition against *Vibrio harveyi*. WD, WA and FS are other bacteria isolated from biofloc culture system that did not exhibit any lytic activity against *Vibrio harveyi*

Table 1: Reactions of bacteria on the 29 dehydrated substrates of BBL Crystal™ Identification Kit

Panel location	Substrates	Results
4A	Florescent negative control	*
2A	4 MU-β-D-glucoside	+
1A	L-valive-AMC	+
4B	L-phenylalanine	+
2B	4 MU-α-D-glucoside	-
1B	L-pyroglutamic acid-AMC	+
4C	L-tryptophan	+
2C	L-arginine-AMC	-
1C	4 MU-N-acetyl-β-D-glucosaminide	+
4D	4 MU-phosphate	+
2D	4 MU-β-D-glucoronide	-
1D	L-isoleucine-AMC	-
4E	Trehalose	-
2E	Lactose	-
1E	Methyl-α and β-glucoside	-
4F	Sucrose	-
2F	Mannitol	-
1F	Maltoriose	-
4G	Arabinose	-
2G	Glycerol	-
1G	Fructose	-
4H	p-n-p-β-D-glucoside	-
2H	p-n-p-β-D-cellobioside	-
1H	Proline and Leucine-p-nitroanilide	+
4I	p-n-p-phosphate	+
2I	p-n-p-α-D-maltoside	-
1I	ONPG and p-n-p-α-D-galactoside	-
4J	Urea	+
2J	Esculin	-
1J	Arginine	+

The *in vivo* competitive interaction against *V. harveyi* showed that there is a significantly lower counts *V. harveyi* in the hepatopancreas of shrimp in the treatment that received *M. luteus* inoculum (T2) addition as compared to the treatment that received only *V. harveyi* inoculum (T3)

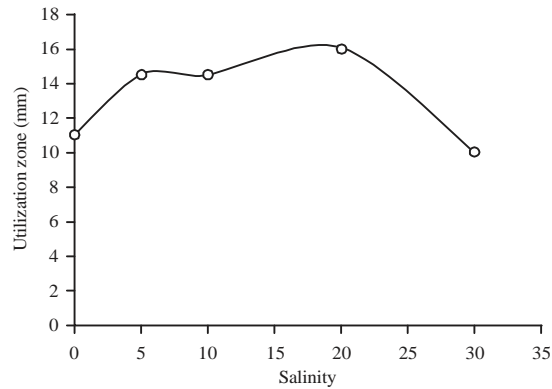


Fig. 2: Zone of utilization of *Micrococcus luteus* in *Vibrio harveyi* dead cells at different salinities

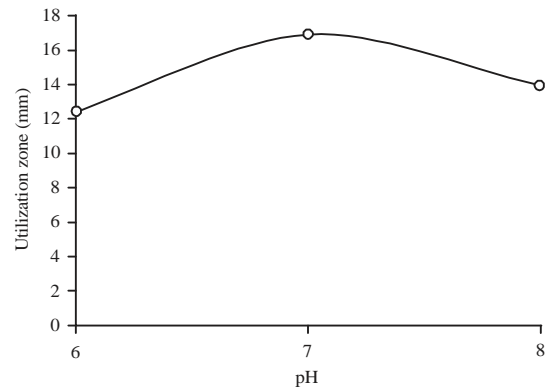


Fig. 3: Zone of utilization of *Micrococcus luteus* in *Vibrio harveyi* dead cells at different pH levels

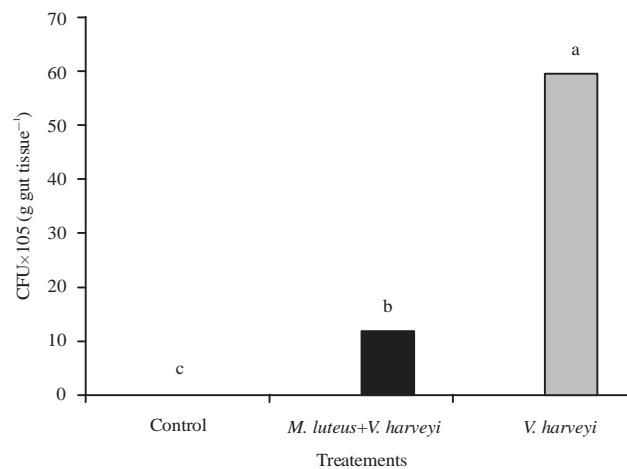


Fig. 4: Recovered total *Vibrio harveyi* count from the different treatments

(Fig. 4). The total *V. harveyi* count for T2 (*M. luteus*+*V. harveyi*) and T3 (*V. harveyi* alone) were  $12 \times 10^5$  and  $59.5 \times 10^5$  colony forming unit g gut tissue<sup>-1</sup>, respectively. The control was found negative with *V. harveyi*.

## DISCUSSION

Results of the present study indicate that the inhibitory activity of Biofloc culture system against *V. harveyi* could be attributed to the presence of *Micrococcus luteus* exhibiting strong lytic activity against this pathogenic *Vibrio*. The antagonistic property of the isolated *M. luteus* bacteria could be due to the secretions of compounds that are toxic (bactericidal) or inhibitory (bacteriostatic) towards other microorganisms (Lara-Flores, 2011). This antibacterial effect attributed to the combination or single action of antibiotics, bacteriocins, siderophores, lysozymes, proteases and/or hydrogen peroxide and the alteration of pH values by the production of organic acids (Verschuere *et al.*, 2000).

The mechanism by which *M. luteus* exerts its anti-*Vibrio* activity was evaluated by testing the ability of this isolate to lyse the cells of *V. harveyi*. Results indicate that *M. luteus* is capable of lysing cells of *V. harveyi*. Further this activity is exhibited in a wide range of salinity from 0-30 ppt with optimum lytic activity observed at around 20 ppt. Lytic activity was also found to be active at a pH range of 6-8. This indicates that the bacteria could elicit its anti-*Vibrio* activity both inside the gut of the host shrimp with neutral or slightly acidic pH and at the water with pH range of about 8. *Micrococcus* is an ubiquitous microbe that is almost known to be present in almost all types of habitats. Although, there exist a good record of earlier reports elucidating the use of *M. luteus* in the production of industrial compounds and its industrial applications but information on the antagonistic activity of this bacteria against shrimp pathogens has been scarce.

The strong cellular lytic activity of *M. luteus* isolate on cells of *V. harveyi* in the present study suggests the utilization of *V. harveyi* cells as nutrient by *M. luteus*. Wahid *et al.* (2007) stated that the cellular lytic activity is a strategy used by microbes to extract the needed nutrient from other microorganism that died from competition. The findings of the present study is comparable to the work of Rattanachauy *et al.* (2010) showing that the Anti-*Vibrio* activity of the *Pseudomonas* sp. is manifested by killing the *Vibrio* with the production of secondary metabolites and lysis of the cells with extracellular proteases. This inhibitory strategy is employed by *Pseudomonas* sp. to eliminate its *Vibrio* competitor and utilize it as a nutrient source.

Further findings of the present study concur with the findings of Jayaprakash *et al.* (2005) in another isolate of *Micrococcus* (MCCB 104). The study demonstrated extracellular enzymes are responsible for the antagonistic properties of this isolate against *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, *V. fluviallis*, *V. nereis*, *V. proteolyticus*, *V. mediterranei*, *V. cholerae* and *Aeromonas* sp. Kafilzadeh *et al.* (2014) also reported an isolate of *Micrococcus luteus* to have *in vitro* antagonistic property against *Vibrio parahaemolyticus*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella* sp. and *Vibrio cholerae* indicating the strong antagonistic activity against gram negative pathogens.

The ability of the bacteria to colonize and establish in the host's gut is considered as an important factor in prevention of bacterial pathogen entrance and establishment to the host organism (Verschuere *et al.*, 2000). In the present study, the isolated *M. luteus* was found to exhibit better hepatopancreas colonizing activity than the pathogenic *V. harveyi*. Further shrimp inoculated with *M. luteus* and introduced *V. harveyi* exhibited a much lower count of *V. harveyi* in the hepatopancreas. In accordance to the findings of the present study, the work of Abd El-Rhman *et al.* (2009) elucidated that *M. luteus* isolated in tilapia intestines are able to inhibit *Aeromonas hydrophila* colonization to the gut of Tilapia. Other species of *Micrococcus* have also been shown to exhibit probiotic potential in inhibiting pathogenic bacteria colonization in the gut as reported in juvenile *L. vannamei* (Kafilzadeh *et al.*, 2014) and in *Macrobrachium rosenbergii* larvae (Jayaprakash *et al.*, 2005).

## CONCLUSION

The present study provide evidence that the *Vibrio* inhibiting activity of biofloc culture system could be due to *Micrococcus luteus*. This bacteria exhibited a potent *V. harveyi* lytic activity and a strong hepatopancreas colonizing activity.

## ACKNOWLEDGMENT

The authors are grateful for the financial support provided by the Office of the Vice Chancellor for Research and Extension (OVCRE) of the University of the Philippines Visayas, Miag ao, Iloilo and the Department of Science and Technology Accelerated Science and Technology Human Resource Development Program (DOST ASTHRDP).

## REFERENCES

- Abd El-Rhman, A.M., Y.A.E. Khattab and A.M.E. Shalaby, 2009. *Micrococcus luteus* and *Pseudomonas* species as probiotics for promoting the growth performance and health of Nile tilapia, *Oreochromis niloticus*. *Fish Shellfish Immunol.*, 27: 175-180.
- Avnimelech, Y., 2007. Feeding with microbial flocs by tilapia in minimal discharge bio-flocs technology ponds. *Aquaculture*, 264: 140-147.
- Ballester, E.L.C., P.C. Abreu, R.O. Cavalli, M. Emerenciano, L. De Abreu and W. Wasielesky Jr., 2010. Effect of practical diets with different protein levels on the performance of *Farfantepenaeus paulensis* juveniles nursed in a zero exchange suspended microbial flocs intensive system. *Aquacult. Nutr.*, 16: 163-172.
- Cadirci, B.H. and S. Citak, 2005. A comparison of two methods used for measuring antagonistic activity of lactic acid bacteria. *Pak. J. Nutr.*, 4: 237-241.
- Crab, R., Y. Avnimelech, T. Defoirdt, P. Bossier and W. Verstraete, 2007. Nitrogen removal techniques in aquaculture for a sustainable production. *Aquaculture*, 270: 1-14.
- De la Peña, L.D., C.R. Lavilla-Pitogo, A. Namikoshi, T. Nishizawa, Y. Inui and K. Muroga, 2003. Mortality in pond-cultured shrimp *Penaeus monodon* in the Philippines associated with *Vibrio harveyi* and white spot syndrome virus. *Fish Pathol.*, 38: 59-61.
- FAO., 2009. The State of World Fisheries and Aquaculture 2008. FAO Fisheries and Aquaculture Department, Food and Agriculture Organization, Rome, Italy, ISBN: 978-92-5-106029-2, Pages: 196.
- FAO., 2014. Cultured aquatic species information programme: *Penaeus vannamei* (Boone, 1931). FAO Fisheries and Aquaculture Department, Food and Agriculture Organization, Rome, Italy.
- Gullian, M., F. Thompson and J. Rodriguez, 2004. Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*. *Aquaculture*, 233: 1-14.
- Hargreaves, J.A., 2013. Biofloc production systems for aquaculture. Southern Regional Aquaculture Center, pp: 1-12.
- Jayaprakash, N.S., S.S. Pai, A. Anas, R.Preetha, R. Philip and I.S.B. Singh, 2005. A marine bacterium, *Micrococcus* MCCB 104, antagonistic to vibrios in prawn larval rearing systems. *Dis. Aquat. Org.*, 68: 39-45.
- Kafilzadeh, F., M. Ziarati and M.A. Keysami, 2014. Putative bacterial flora assessment in juvenile *Litopenaeus vannamei* as probiotics by using antibacterial activity tests. *Biosci. Biotechnol. Res. Asia*, 11: 507-515.
- Karunasagar, I., R. Pai, G.R. Malathi and I. Karunasagar, 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. *Aquaculture*, 128: 203-209.



- Kesarcodi-Watson, A., H. Kaspar, M.J. Lategan and L. Gibson, 2008. Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. *Aquaculture*, 274: 1-14.
- Lara-Flores, M., 2011. The use of probiotic in aquaculture: An overview. *Int. Res. J. Microbiol.*, 12: 471-478.
- Rattanachuay, P., D. Kantachote, M. Tantirungkij, T. Nitoda and H. Kanzaki, 2010. Inhibition of shrimp pathogenic vibrios by extracellular compounds from a proteolytic bacterium *Pseudomonas* sp. W3. *Electron. J. Biotechnol.*, Vol 13. 10.2225/vol13-issue1-fulltext-2
- Sivakumar, N., M. Sundararaman and G. Selvakumar, 2012. Probiotic effect of *Lactobacillus acidophilus* against vibriosis in juvenile shrimp (*Penaeus monodon*). *Afr. J. Biotechnol.*, 11: 15811-15818.
- Than, P.P., C.S. Del Castillo, T. Yoshikawa and T. Sakata, 2004. Extracellular protease production of bacteriolytic bacteria isolated from marine environments. *Fish. Sci.*, 70: 659-666.
- Verschuere, L., G. Rombaut, P. Sorgeloos and W. Verstraete, 2000. Probiotic bacteria as biological control agents in aquaculture. *Microbiol. Mol. Biol. Rev.*, 64: 655-671.
- Wahid, M.I., T. Yoshikawa and T. Sakata, 2007. Bacteriolytic activity and growth of marine isolates of labyrinthulids on dead bacterial cells. *Fish. Sci.*, 73: 1286-1294.
- Wasielesky, Jr. W., H. Atwood, A. Stokes and C.L. Browdy, 2006. Effect of natural production in a zero exchange suspended microbial floc based super-intensive culture system for white shrimp *Litopenaeus vannamei*. *Aquaculture*, 258: 396-403.