Bangladesh J. Zool. **38**(1): 41-49, 2010

ABUNDANCE AND COMMUNITY STRUCTURE OF BACTERIA ASSOCIATED WITH *ARTEMIA* CYSTS COMMONLY USED IN SHRIMP HATCHERY OF BANGLADESH

Tazin Fahmi, M. Manjurul Karim, Shafiqur Rahman,
M. Niamul Naser[1] and Shakila N. Khan*

Department of Microbiology, University of Dhaka, Dhaka-1000, Bangladesh

Abstract: The abundance and community structure of microorganisms associated with the commercial brands of brine shrimp, *Artemia,* cysts were studied in a microbiologically-monitored controlled environment for hatching the cysts. The cysts, treated with chlorine, showed no presence of microorganisms during the pre-hatching stage, contrary to those which were untreated; indicating the surface adhering of microorganisms with the cysts. Interestingly, microorganisms were isolated from both the chlorine-treated and untreated cysts when they were hatched. The isolated microorganisms were identified by microscopic, biochemical and cultural properties, and some of them were found potentially pathogenic. This indicates the association of bacteria with *Artemia* during its encapsulation stage, which is released once the cysts are hatched. This is a cause of concern of introducing bacterial diseases in shrimp hatcheries of Bangladesh during feeding the shrimp larvae.

mvi-ms‡¶ct evBb wk¤ú *Artemia*-i wm‡÷i ewYwR¨K evÛ Ges AbRx‡ei msL¨v I KwgDwbwU MV‡bi g‡a¨ m¤úK© wbYq Kiv nq| cwi¯dUb ce †Kwib-†kwaZ wm‡ó †Kvb AYRx‡ei Dcw¯'Z †bB, A_P A‡kwaZ wm‡ó Gi cvPhZv j¶¨Yxq, A_vr AYRxe¸‡jv wm‡ói Mv‡Î Ae¯'vb wb‡q _v‡K| GLv‡b D‡jL¨ ‡h, cwi¯dU‡bi ci A†kwaZ, GgbwK †Kwib-†kwaZ wmó¸‡jv †_‡KI wewfb ai‡bi AYRx‡ei Dcw¯'Z j¶¨ Kiv †M‡Q, hv n¨vPvix cieZx 72 N›Uvi g‡a¨ ixwZgZ wekvj msL¨v avivY K‡i| AvYwe¶wYK I cvY-ivmvqwbK ag¸‡jv cix¶v Kivi ci cwi¯dUb-cieZx wmó¸‡jv †_‡K Ggb wKQ AYRxe‡K mbv³ Kiv †M‡Q hv n¨vPvix wk‡í Drcw`Z cvYx¸‡jvi wewfb ai‡bi †iv‡Mi msµgYmn go‡Ki gZ cfZ ¶wZ mvab Ki‡Z cv‡i| we‡`k †_‡K Avg`vbx Kiv GBme *Artemia* wmó¸‡jvi mv‡_ cwiewnZ AYRxe Ges wPswo n¨vPvix wk‡í Zv‡`i cfve ZvB GKwU D‡`‡Mi welq|

Key words: *Artemia*, TCBS medium, vibrios, shrimp, larvae, hatchery

INTRODUCTION

The use of live feed presents a biosecurity risk for aquaculture due to the introduction of bacterial pathogens. Reared *Artemia* nauplii constitute the most commonly used live feed for larval aquaculture species including fish, crustaceans and mollusks, produced in industrial hatcheries (Sorgeloos *et al.* 1986). Its unique property to form dormant embryos, so called 'cysts', makes it a convenient, long-term storable and suitable feed for the larvae. Several studies have, however, demonstrated that *Artemia* nauplii is a vector responsible for introducing bacteria into hatchery systems (Gilmour *et al.* 1975, Benavente and Gatesoupe 1988) including potentially pathogenic bacteria (Gomez-Gil *et al.* 1994, Lopez-Torres and Lizarraga-Partida 2001). The source of such contamination could be the cultivation of *Artemia* in poor conditions, such as

*Corresponding author. E mail: shakilamanjur@yahoo.com [1]Department of Zoology, University of Dhaka, Dhaka-1000, Bangladesh

using only bacteria as feed (Rico Mora and Voltolina 1995, Makridis *et al.* 2000) or using autoclaved (Orozco-Medina *et al.* 2002) or irradiated inert feed (Verschuere *et al.* 1999, 2000). Such artificial environments in combination with poor feed negatively influence the overall condition of gnotobiotically-grown *Artemia*. Most bacteria are localized in the nauplii gut, with external surfaces having limited bacterial colonization (Høj *et al.* 2009). Therefore, *A.* nauplii are often treated in order to reduce the bacterial load associated with them prior to feeding them to the larvae.

OBJECTIVES

Since Bangladesh does not possess any *Artemia* source in her territory, cysts are imported from many countries, e.g. USA, Taiwan, Thailand and China, and are extensively used in shrimp and prawn hatcheries. Such a usage of live feed may introduce various types of organisms including pathogens, and thereby pose a potential threat to the rearing system. This study is therefore aimed to investigate the abundance and community structure of bacteria associated with the cysts from different brands of *Artemia* under laboratory sterile conditions.

MATERIAL AND METHODS

Samples: Five different commercial brands of *Artemia* cysts were analyzed. These were Red Jungle, USA; Cowboy, USA; Great Salt Lake, USA and two different samples from Sanders, Thailand.

Hatching of Artemia cyst: Experimental design was performed to create a gnotobiotic environment for hatching the cysts of *Artemia*. Sterilized 250 ml cotton-plugged conical flasks were used as bioreactor for hatch operations. Hatching was performed in flasks placed in an orbital shaker shaking at 100 rpm and at 28-30^{o}C with continuous illumination. A set of flasks was introduced with *Artemia* cysts directly from imported cans without any treatment and another set was introduced following disinfection by chemical treatment. A 7% bleach solution prepared in sterile sea water was used to disinfect *Artemia* cysts. All performances were done inside a laminar airflow cabinet. Each sample was checked in triplicate.

Bacteriological analyses: 0.2 g cysts of each sample were inoculated in each hatching flask with 200 ml seawater. Water samples were collected from the hatching flasks at daily intervals from 0 hr up to 72 hr 0.1 ml aliquot of each sample was inoculated by spread plate method on different complex and selective media, viz. nutrient agar (Oxoid, UK), thiosulfate-citrate-bile-sucrose (TCBS) agar (Oxoid), VHA agar (Harris *et al.* 1996), MacConkey agar (Oxoid) and

cetrimide agar (Oxoid), for the enumeration of total viable heterotrophic bacteria, TCBS bacteria, *Vibrio harveyi,* total coliforms and *Pseudomonas,* respectively.

Isolation, purification and conservation of bacterial strains: Seawater was used for all kinds of media preparation. After incubation for 24 hr at 30 ºC, viable bacterial cell count was enumerated on nutrient agar. After counting, randomly selected colonies from the selective media were inoculated by streaking onto nutrient agar. A visual observation of colony and microscopic observation of cells confirm the purification of strain. The purified strains were conserved in screw cap vials containing glycerol broth which is known as stock culture. These stock cultures of bacteria had been stored at 4 ºC until further use. Subculturing on nutrient agar from this stock culture was done for further analysis.

Screening tests: Nine strains were phenotypically selected based on their size, shape and Gram-reaction, and then were subjected to several biochemical tests, such as motility, catalase, oxidase, carbohydrate fermentation, KIA, citrate utilization, salt tolerance, etc. (Sneath *et al.* 1986). From pure culture each strain was also observed for pigmentation.

RESULTS AND DISCUSSION

Bacteriological analysis of *Artemia:* Five imported commercial brands of *Artemia* cysts were incubated at 30 ºC in seawater under illumination for hatching. Water samples were withdrawn at daily intervals for the enumeration of total viable heterotrophic bacterial (VHB) count on different selective media. The chlorine-treated cysts showed no growth on any media at the onset of treatment (i.e. 0 hr); but the cysts without treatment produced significant growth on nutrient agar (Fig. 1). This indicates the presence of bacteria adhering to the surfaces of the cysts, imported directly for local use.

Cyst hatching was observed after 24 hr of incubation. The chlorine-treated cysts that produced no growth from the water sample at the time zero (Fig. 1) yielded countable growth in almost all selective media as soon as the cysts were hatched (Fig. 2). The number of microbial count was found increasing as the hours of incubation progressed, and the count was extremely high after 72-hr post incubation. It indicates that bacteria were encapsulated with *Artemia* when the cysts were formed in otherwise non-sterile environments, and they were released from the dormant cysts once the cysts were hatched, and produced increasing number of bacteria as *Artemia* aged in their different stages of life cycle. Needless to say, we observed rather a higher figure of microbial population

associated with untreated cysts throughout the post-hatching period of *Artemia* (data not shown).

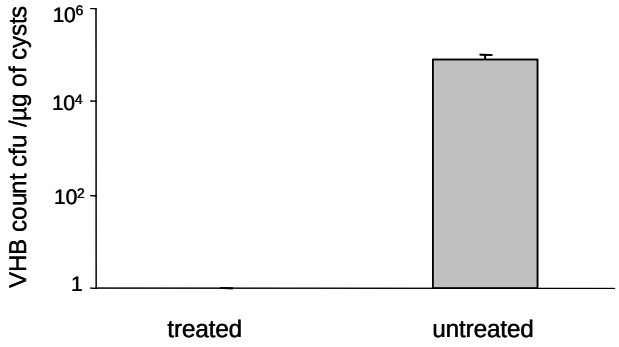

Fig. 1. VHB count in cysts surface. *Artemia* cysts, either treated with chlorine (treated) or not (untreated) were harvested in seawater, and the sample waters were withdrawn for viable heterotrophic bacterial (VHB) counts at time zero on nutrient agar. The counts were averaged from the bacterial counts obtained from five different brands of *Artemia*.

Identification of bacteria: A total of nine colonies isolated randomly from different selective media was picked up for identification. Of them, six colonies were isolated from TCBS, two from MacConkey and one from cetrimide agar. Their microscopic examination and the colony appearance are mentioned in Table 1.

Table 1. Morphology and colony characteristics of some bacteria isolated from hatched *Artemia*.

Colony ID	Morphology	Gram reaction	Colony color
1T	Cocci, form tetrad	Gram positive	Green on TCBS
2T	Grape like structure	Gram positive	Green on TCBS
3T	Short rod	Gram positive	Green on TCBS
4T	Short rod	Gram negative	Light green on TCBS
5T	Curved rod	Gram negative	90% green on TCBS
6T	Short rod	Gram negative	Yellow on TCBS
7M	Large rod	Gram negative	Light pink on MacConkey
8M	Short rod	Gram negative	Deep pink on MacConkey
9C	Rods, straight to slightly curved, monotrichous	Gram negative	Blue green pigmented colony on cetrimide agar

The colonies on TCBS agar, which was used to isolate *Vibrio* spp, a Gram negative bacterium also produced colonies that were Gram positive, as was revealed in the Gram reaction (colonies ID: 1T, 2T and 3T, Table 1). Following the biochemical tests (Table 2), the cocci-shaped 1T and 2T were identified as

Micrococcus sp. and *Staphylococcus* sp., while the bacilli-shaped 3T was recognized as *Bacillus* sp.

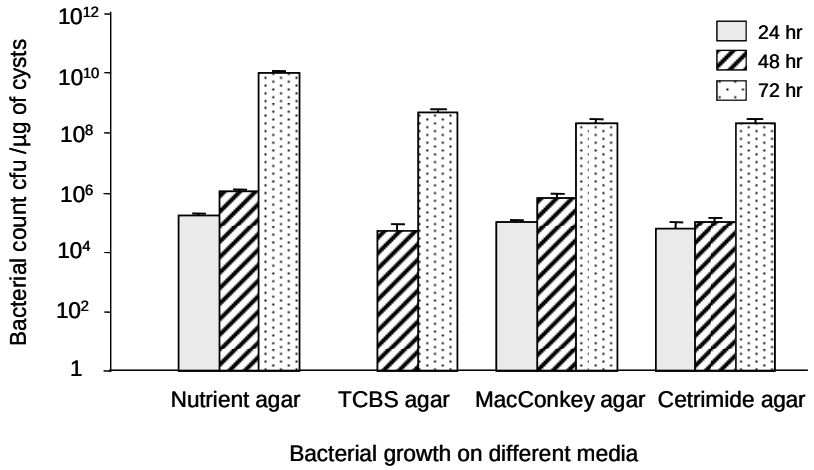

Fig. 2. Bacterial count from hatched *Artemia*. The chlorine-treated *Artemia* cysts were harvested in seawater for hatching, and the sample waters were withdrawn at daily intervals for bacterial counts on different selective media, e.g. nutrient agar, TCBS agar, MacConkey agar and cetrimide agar. The counts were averaged from the bacterial counts obtained from five different brands of *Artemia*.

Table 2. Biochemical characteristics of bacteria isolated on TCBS media of hatched *Artemia*.

Colony ID	1T	2T	3T	4T	5T	6T
Growth on TCBS	+	+	+	+	+	+
H_2S	-	-	-	-	-	-
Oxidase	-	-	-	+	+	+
Catalase	+	+	-	+	-	-
Glucose	-	+	+	+(A)	+	+
Sucrose	-	A	+	+(A)	-	+
Mannose	-	-	-	+	+	+
Mannitol	-	+	+	+	+	-
Gelatinase	+	+	+	-	+	+
Arabinose	-	+	-	-	V	-
Cellobiose	-	-	-	+	V	-
Ornithine	-	-	-	+	-	+
Citrate	-	+	+	-	+	+
Indole	-	-	-	+	+	+
Growth on 0% NaCl	+	+	+	-	-	-
Growth on 6.5% NaCl	-	+	+	+	+	+
Lysine	-	-	-	+	+	+
Arginine	+	-	-	-	-	-

A=acid; V=variable.

The Gram negative colonies on TCBS agar on the other hand, were looked for the identification of *Vibrio* spp. in a series of biochemical tests that were based on the A/L/O (arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase) dendogram, as proposed by Alsina and Blanch (1994) (Table 2). Colonies 4T and 6T were identified as *V. harveyi* and *V. alginolyticus,* respectively. This identification was further complemented when they were subjected to grow on VHA medium, where they produced light green colonies with dark center; and blue color respectively, traits which were typical for *V. harveyi* and *V. alginolyticus*, respectively in VHA medium (Harris *et al.* 1996). The colony 5T was primarily identified as *V. campbellii*.

The Gram negative colonies, 7M and 8M that grew on MacConkey agar were primarily identified as *Klebsiella* sp. and *Escherichia coli,* respectively based on the results they produced in biochemical tests (Table 3). Further, colony 7M produced characteristic light pink pigmentation in MacConkey agar, which is suggestive for being *Klebsiella*. The colony 9C that grew on selective medium, Cetrimide agar produced blue-green pigmentation, and exhibited Gram negative reaction, and positive reactions to catalase and oxidase tests (Table 3). Therefore, the colony was identified as *Pseudomonas* sp.

Table 3. Biochemical characteristics of bacteria isolated on cetrimide and MacConkey media of hatched Artemia cysts.

Colony ID	7M	8M	9C
Catalase	+	+	+
Oxidase	-	-	+
MR	+	+	-
VP	+	+	-
Indole		+	-
Motility	-	+	+
Citrate	-	+	+
Urease	+	-	+

The culture of aquatic organisms is often hampered by the occurrence of unpredictable diseases even a good controlled hatching condition is maintained. A large portion of aquaculture species around the world are given *Artemia* nauplii as their feed in early life stages (Sorgeloos *et al.* 1986). Ironically, *Artemia* have the capacity for bioencapsulation of bacterial cells during cyst formation (Makridis *et al.* 2000, Verschuere *et al.* 2000), hence, the careless use of this widely used live feed may introduce pathogens to the aquaculture which could be responsible for the diseases and mortalities. In Bangladesh, *Artemia* is a popular feed in shrimp aquaculture system, and a very little is known about its microbiology. Most of causing farmers use the canned feed directly, not treated with chlorine in the hatcheries. After examining five commercial brands of

Artemia routinely used in Bangladesh, we observed that the untreated cysts from cans can introduce a great number of microorganisms into the system. The treatment with chlorine was, however, able to eliminate the microflora, estimated about one million bacteria per microgram of cysts on an average, thought to be present at the cysts surface (Fig. 1). This study also noticed that chlorine treatment increased the hatching rate of the cysts. Hence, it is a good practice of sanitizing the cysts before it is delivered as a feed.

Cysts, be it sanitized or not, produced a heavy bacterial load following their hatching over a period of 72-hours (Fig. 2). The count was progressively larger with days as *Artemia* matures to nauplii. This indicates that microorganisms are bioencapsulated with *Artemia* cysts that are released following their hatching, and the pretreatment with chlorine has got no effect on them. Presence of these organisms in *Artemia* cysts clearly indicates that *Artemia* cysts can act as a vector to introduce a large number of bacteria, possibly allochthonous in origin, hence are being intruded in our aquaculture system through the feeding process. In order to address whether the bioencapsulated bacteria are pathogenic or not, we attempted to identify some of the isolates collected from TCBS, MacConkey and Cetrimide agar media. After a series of microscopic, cultural and biochemical examinations, some of the isolates were identified as *V. harveyi and V. alginolyticus,* the agents that pose a threat for vibriosis, which is frequently associated in shrimp aquaculture with high mortality and severe economic loss in all producing countries (Lightner 1988). On TCBS media, which is selective for *Vibrio* spp. a Gram negative bacterium, we were also able to detect Gram positive bacteria, viz. *Bacillus, Micrococcus* and *Staphylococcus* spp. This was well consistent with the finding by Lopez-Torres and Lizarraga-Partida (2001).

The bacterial population observed under laboratory condition showed that the vibrios introduced with *Artemia* nauplii as a vector came from the cysts which might have been trapped during bioencapsulation. *Vibrio* spp. was not evident after a 24 h incubation, but became dominant after 48 h (Fig. 2), probably because during hatching, *Artemia* cysts are broken and a reserve organic substance, glycerol, is excreted to hatching water (Sorgeloos *et al.* 1986). Glycerol is an organic substrate that is utilized efficiently by *Vibrio* spp. (Bianchi 1976). A very low inoculum of this population could become dominant, utilizing glycerol rather than the Gram-positive population. Wen-Yu *et al.* (1994) have reported a bacterial population change, from Gram-positive to Gram-negative bacteria since the introduction of *Artemia* nauplii as food for *Penaeus monodon* larvae, at the Zoea stage III.

It is therefore, necessary to control the bacterial population of nauplii to minimize the danger of bacterial infection before their use in culture system. Many techniques, such as antibiotics, chemicals like formaldehyde, ultraviolet treatments, freezing have been investigated to minimize the danger of bacterial infections associated with feeding live food (Gilmour *et al.* 1975, Hameed and Balasubramanian 2000, Coleman *et al.* 1980, Hatai *et al.* 1981). However, use of chemotherapeutics and chemicals has led to the emergence of more virulent pathogens (Hameed and Balasubramanian 2000) with the eventual transfer of antimicrobial resistance to human pathogens (Karunasagar *et al.* 1994). The present study observes that the hatching process of *Artemia* cysts could be a key point for the control of pathogenic bacteria in shrimp hatcheries. The introduction of probiotic bacteria rather than antibiotics could be a better way of controlling bacterial infections (Rahman *et al.* 2009).

CONCLUSIONS

The present study was carried out to determine the microbial status of the *Artemia* cysts imported for local use in the hatcheries and grow out systems of shrimp and fish aquaculture as a feed in Bangladesh. Following hatching, the cysts, be it sanitized or not, liberate a great number of microorganisms in the culture system, indicating the entrapment of microbes during cyst formation of *Artemia*. Importantly, some of the microbes have the potentiality to cause various bacterial diseases in fish, as identified in our study. Further study is therefore needed to control the microorganisms, bioencapsulated in cysts, and their effect in animals of aquaculture.

LITERATURE CITED

ALSINA, M. and BLANCH, A.R. 1994. A set of keys for biochemical identification of environmental *Vibrio* species. *J. Appl. Bacteriol.* **76**: 79-85.

BENAVENTE, G.P. and GATESOUPE, F.J. 1988. Bacteria associated with cultured rotifers and *Artemia* are detrimental to larval turbot, *Scophthalmus maximus* L. *Aquac. Eng.* **7**: 289-293.

BIANCHI, M. 1976. Etude taxonomique et distribution e´cologique des bacte´ries vibrio¨ıdes du milieu marin. The´se Dr. Sc. Nat. Universite´Aix-Marseille II, 126 pp.

COLEMAN, D.E., NAKAGAWA, L.K., NAKAMURA, R.M. and CHANG, E. 1980. In: *The Brine Shrimp,* Vol. **3**. (Eds., Persoone, G., Sorgeloos R.P., Jaspers, E.) Universa Press Wetteren, Belgium, p. 153.

GILMOUR, A., McCALLUM, M.F. and ALLAN, M.C. 1975. Antibiotic sensitivity of bacteria isolated from canned eggs of the California brine shrimp (*Artemia salina*). *Aquaculture* **6**: 221-231.

GOMEZ-GIL, R.S.B., ABREU-GROBOIS, F.A., ROMERO-JARERO, J. and HERRERA-VEGA, M. 1994. Chemical disinfection of *Artemia* nauplii. *J. World Aquacult. Soc.* **25**(4): 579-583.

HAMEED, A.S.S and BALASUBRAMANIAN, G. 2000. Antibiotic resistance in bacteria isolated from *Artemia* nauplii and efficacy of formaldehyde to control bacterial load. *Aquaculture* **183**: 195-205.

HARRIS, L., OWENS, L. and SMITH, S. 1996. A selective and differential medium for *Vibrio harveyi*. *Appl. Environ. Microbiol.* **62**(9): 3548-3550.

HATAI, K., YASUMOTO, S., OGAWA, S. and YASUNAGA, N. 1981. Elimination of bacteria associated with baking yeast-fed rotifers by some antibacterial agents. *Rep. Nagasaki Prefect. Fish. Stn.* pp. 218-222.

HØJ, L., BOURNE, D.G. and HALL, M.R. 2009. Localization, abundance and community structure of bacteria associated with *Artemia*: Effects of nauplii enrichment and antimicrobial treatment, *Aquaculture* **293**: 278-285.

KARUNASAGAR, I., PAI, R., MALATHI, G.R. and KARUNASAGAR, I. 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. *Aquaculture* **128**: 203-209.

LIGHTNER, D.V. 1988. *Vibrio* disease of penaeid shrimp. In*: Disease diagnosis and control in North American marine aquaculture.* (Ed., SINDERMANN C.J. and LIGHTNER, D.V.) Elsevier, Amsterdam, The Netherlands. pp. 42-47.

LOPEZ-TORRES, M.A. and LIZARRAGA-PARTIDA, M.L. 2001. Bacteria isolated on TCBS media associated with hatched *Artemia* cysts of commercial brands. *Aquaculture* **194**: 11-20.

MAKRIDIS, P., FJELLHEIM, A., SKJERMO, J. and VADSTEIN, O. 2000. Control of the bacterial flora of *Brachionus plicatilis* and *Artemia franciscana* by incubation in bacterial suspensions. *Aquaculture* **185**: 207-218.

OROZCO-MEDINA, C., MAEDA-MARTÍNEZ, A. and LÓPEZ-CORTÉS, A. 2002. Effect of aerobic Gram-positive heterotrophic bacteria associated with *Artemia franciscana* cysts on the survival and development of its larvae. *Aquaculture* **213**: 15-29.

RAHMAN, S., KHAN, S.N., NASER, M.N. and KARIM, M.M. 2009. Application of probiotic bacteria: a novel approach towards ensuring food safety in shrimp aquaculture. *J. Bangladesh Acad. Sci.* **33**(1): 139-144.

RICO-MORA, R. and VOLTOLINA, D. 1995. Effects of bacterial isolates from *Skeletonema costatum* cultures on the survival of *Artemia franciscana* nauplii. *J. Invertebr. Pathol.* **66**: 203-204.

SORGELOOS, P., LAVENS, P., LE´GER, P., TACKAERT, W. and VERSICHELE, D. 1986. *Manual for the culture and use of brine shrimp Artemia in aquaculture.* The Belgian Administration for Development Cooperation. United Nations Food and Agriculture Organization, Belgium. 319 pp.

SNEATH, P.H.A., MAIR, N.S., SHARPE, M.E. and HOLT, J.G. (Eds.) 1986. *Bergey's Manual of Systemic Bacteriology.* vol. 2. Williams and Wilkins, Baltimore, pp. 1599.

VERSCHUERE, L., HEANG, H., CRIEL, G., SORGELOOS, P. and VERSTRAETE, W. 2000. Selected bacterial strains protect *Artemia* spp. from pathogenic effects of *Vibrio proteolyticus* CW8T2. *Appl. Environ. Microbiol.* **66**(3): 1139-1146.

VERSCHUERE, L., ROMBAUT, G., HUYS, G., DHONT, J., SORGELOOS, P. and VERSTRAETE, W. 1999. Microbial control of the culture of *Artemia* juveniles through pre-emptive colonization by selected bacterial strains. *Appl. Environ. Microbiol.* **65**(6): 2527-2533.

WEN-YU, L., SHIU-NAN, C. and GUANG-HSIUNG, K. 1994. Studies of the flora variation of heterotrophic bacteria in giant tiger prawn (*Penaeus monodon*) hatchery. *Coa. Fish Ser.* **47**: 1-20.

(Manuscript received on November 7, 2009; revised on December 7, 2009)