A putative probiotic isolated from hatchery reared juvenile *Penaeus monodon*

Shakibazadeh S. [1`], Saad Che Roos [1*], Hafezieh M.[2], Christianus A.[1], Saleh Kamarudin M. [1], Sijam Kamaruzaman [3]

Received: March 2012 Accepted: September 2012

Abstract

Probiotic is considered as a remedy to solve disease problem in shrimp aquaculture. Shrimp body and environment bacterial flora are recommended as reliable source for extracting of beneficial bacteria. Therefore, 5000 pieces of post larvae of *Penaeus monodon* were distributed equally in 4 tanks in probiotic and antibiotic free condition up to juvenile. Then microbiological samples from digestive system, muscles, body surface, rearing water and tank sediment were inoculated onto the selective media plates. Incident of total bacteria, *Vibrio*, *Pseudomonas* and *Entrobacteriaceae* were evaluated in different body parts and rearing environment of shrimp. The highest bacteria count for total bacteria, *Vibrio*, *Pseudomonas* was occurred in sediment followed by digestive system. No *Entrobacteriaceae* was observed from all samples. Isolated bacteria were identified and placed in 7 genera consisted of *Vibrio*, *Shewanella*, *Burkholderia*, *Clavibacter*, *Staphylococcus*, *Brevibacterium*, *Corynebacterium*, while one genus remained unidentified. Genus *Shewanella* exhibited highest frequent bacteria in digestive system of *P. monodon* which may be indicated its ability to colonization in digestive system. *Shewanella algae* was selected from whole isolated bacteria as candidate probiotic via well plate diffusion technique and cross streak antagonistic test against *Vibrio harveyi*, *V. parahaemolyticus* and *V. alginolyticus*. It was able to digest lipid and protein in tributyrin agar and skim milk agar.

Keywords: Probiotic, *Shewanella algae*, *Penaeus monodon*, Shrimp

1- Department of aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

2-Iranian fisheries Research Organization, Tehran, Iran

3 -Department of Plant Protection, Universitii Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

Corresponding author's email: cheroos@agri.upm.edu.my

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[DOR: 20.1001.1.15622916.2012.11.4.12.4]

Introduction

Epidemics of bacterial diseases which are occurred in all shrimp growth stages have been caused significant economical failures in many countries (Vaseeharan and Ramasamy, 2003a; Kakoolaki et al., 2010, 2011). Dominant group of bacteria in marine ecosystem are Gram negative bacteria in which *Vibrio* spp. are the most common groups (Brisou et al., 1965), These are typically consists of major normal bacterial flora of both cultural and wild penaeid shrimps (Vanderzant et al., 1971; Costa et al., 1998). In consequence of great importance of ecosystem friendly activities, an increasingly research activities resumed to substitute alternative methods with the conventional disease treatment techniques. Because those are associated with contamination of aquatic ecosystem and increasing of human risks due to consuming of such products (Wang et al., 2005a).

Nowadays, utilization of beneficial microorganisms in aquaculture settings is broadly acknowledged strategy for disease management in compare with the antibiotic therapy and consuming disinfectant compounds (Mohanty et al., 1993, 1996; Gatesoupe, 1999; Gomez-Gil et al., 2000; Sharma and Bhukhar, 2000; Irianto and Austin, 2002; Vine et al., 2006; Wang and Xu, 2006; Wang, 2007).

The most respected specification of a candidate probiotic is its antagonistic abilities against host pathogenic bacteria (Jöborn *et al*., 1997; Rattanachuay, 2011), This may occur due to competitive exclusion via antagonizing the potential pathogen by production of inhibitory compounds or competition for nutrients, adhesion sites or oxygen in the digestive tract (Fuller, 1987). Therefore, *in vitro* antagonistic techniques are utilized as primary step for probiotic screening (Chythanya et al., 2002; Vaseeharan and Ramasamy, 2003a; Ravi et al., 2007; del Castillo, 2008).

Competition for adhesion site between beneficial bacteria and pathogenic bacteria is a possible mechanism for preventing of colonization of pathogenic bacteria onto the digestive system or body surface of the host. Colonization of a beneficial bacteria onto the fish intestine is merely links with its capability to adhere to intestinal mucus and surface(Westerdahl, 1991; Olsson, 1992). *In vitro* adhesion capability of 2 fish pathogens, *V. anguillarum* and *A. hydrophyla*, onto the intestinal epithelium and enteric mucus has been documented (Krovacek et al., 1987; Garcia et al., 1997). It is possible that colonized beneficial bacteria onto the intestinal mucus of hosts' organisms and reveals their protection abilities against pathogens via production of inhibitory substances (Verschuere et al., 2000b).

Contribution of a complement of digestive enzyme producing microorganisms in digestive tract of adult *Penaeus chinensis*, which was assisted to food digestion and producing of some necessary substances has been reported (Wang et al., 2000). Activity of digestive enzyme such as, protease, lipase, amylase in some ontogenetic stages of *Litopenaeus vannamei* was significantly increased when *Bacillus coagulans* SC8168 administered to rearing water of larval

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tanks (Zhou et al., 2009). Administration of a commercial Bacillus to different stages of *Fenneropenaeus indicus* which were consisted of nauplius, zoea, mysis, post larvae and grow out pond considerable difference with the control in terms of amylase, lipase and total protease. Moreover the treatment groups were revealed significantly higher survival rate and wet weight in compare with control (Ziaei-Nejad et al., 2006).

This study was conducted to evaluate the bacterial flora associated with *Penaeus monodon* juveniles which were reared in hatchery at probiotic and antibiotic free condition. The putative isolated bacteria were then evaluated in terms of identification, antagonistic ability, and digestive enzyme production as primary screening tools for potential useful probiotic.

Materials and methods

Culturing condition

Since commercial farm most often use probiotics and/or antibiotics in their rearing procedure which can be caused of bias in the screening process. The study was begun with purchasing of 5000 pieces of apparently healthy post larvae of *P. monodon* (PL_{12}) from a local hatchery. The seeds were placed (1250 pieces/tank) and reared in 4 identical circular fiber glass tanks (2m diameter and 1m height) in the Hatchery complexUniversiti Putra Malaysia. The water was added to the tank at early stage, then it was continued with daily exchanging up to 50%. They were feed with probiotic free commercial pellet for 2 months before being used for bacterial flora sampling. During rearing period pH, salinity and temperature was monitored daily using a pH-meter (YSI, USA) and a hand refractometer (Atago 8808, Japan).

Sampling

Twenty five juvenile shrimps were arbitrarily scooped out from each rearing tank. Those were aseptically dissected and digestive systems (hepatopancreas and intestine) and muscle of juvenile shrimps of each tank were pooled together before being weight. The pooled samples were homogenized and diluted up to 10^{-6} in sterile normal saline. Rearing water of each shrimp tank was collected, homogenized, and serially diluted up to 10^{-4} with sterile normal saline. Sediment sample of each tank was aseptically collected and 10 ml of it was homogenized and serially diluted up to 10^{-8} in sterile normal saline.

Bacteriological study of shrimp body surface was performed after randomly scooping out 25 juvenile shrimp from each rearing tank. Samples were put in a 250 ml Erlenmeyer flask containing 50 ml of sterile normal saline followed by vigorously shaking for 5 min, the wash down normal saline was diluted up to 10^{-4}.

Inoculation and bacterial culture

Four different bacteriological media, TSA (tryptone soy agar), MacConkey agar, TCBS (thiosulphate citrate bile salt agar), and *Pseudomonas*-isolating agar used for selecting and counting of total aerobic heterotrophic bacteria or total viable counts (Xu et al., 1982), genus *Entrobacteriaceae* (Baudart et al., 2005), genus *Vibrio* (McCormack et al., 1974; Xu et al., 1982)and *Aeromonas*, and genus *Pseudomonas* (Krueger and Sheikh, 1987),

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respectively. Then different diluted samples from body parts, rearing water and sediment were cultured onto the cited selected media via spread plate technique.

Isolation and identification of shrimp putative bacteria

Isolation of the bacteria was performed based on their pigmentation, morphological specification and representative category of the colonies which were consisted of at least 10% of the total number of colonies on the subjected plate. The isolated bacteria were then pure cultured, concentrated and kept in LB broth (Defco, USA) containing 15% glycerol at –80°C. Bacterial isolates were subjected to conventional biochemical identification methods such as; Gram stain, Colony morphology, Oxidative, Fermentive, Motility, Oxidase, Catalase, H_2S, Indol, Citrate, MR (Methyl Red), VP (Voges-Proskauer), Nitrate reduction, Urease, Gelatinase, Lysine decarboxylase, Ornithine decarboxyl, Arginine dehydrolyase, Manitol, Glucose, Maltose, Inositol, Sucrose, Lactose, Sorbitol, and Arbinose according to Drew et al. (1986), MacFaddin et al. (1980), and Buchanan (1984). The results were then established via Biolog GN and GP microplates (Biolog, Hayward, CA, USA) as described formerly by Shakibazadeh et al. (2009) consistent with Olsson et al. (2004).

Source of Shrimp pathogens

Vibrio harveyi, ATCC 14126 (American type culture collection), was purchased. Two other shrimp pathogens, *Vibrio parahaemolyticus* and *Vibrio alginolyticus*, were previously isolated from a morbid shrimp and identified as explained before.

Assessment of antagonistic effect against shrimp pathogens

Antagonistic effect of isolates were primarily accessed via Well diffusion plate technique, all isolates were cultured in Mueller Hinton broth containing 2% NaCl for 24 h. The cell free supernatants of each isolate after centrifuging and filter sterilizing were applied as antagonistic agents. Mueller Hinton agar plate supplemented with 2% NaCl which was previously inoculated with 0.5 MacFarland standards of shrimp pathogens, *V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus*, before being punched to form 4mm diameter well for dispensing 50 µl of cell free supernatant of isolates together with normal saline and tetracycline (0.2 µg/µl) as negative and positive controls.

Antagonistic effect of isolates which were formerly exhibited the best inhibitory zone against mentioned shrimp pathogens through Well plate technique were subjected to Cross streak method for further confirming study (Chythanya et al., 2002). Overnight culture of isolates in Mueller Hinton broth (Difco, USA) supplemented with 2% NaCl were inoculated in 2 cm wide bands in diameter of Mueller Hinton agar plates included 2% NaCl (Difco, USA). After 24 h incubation of the inoculated plates at 30°C, the grown 2 cm wide band of isolates were scraped using a sterile slide then the residual of bacteria were killed via exposing to chloroform gas for 15 min. Overnight cultures of three cited shrimp pathogens were adjusted to 0.5 MacFarland standard using a spectrophotometer (Thermospectronic, Genesys 20, USA) and streaked vertically to the previously

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cultured band of tested isolate. Sterile normal saline and overnight culture of isolate adjusted to 0.5 MacFarland standard were used as negative and positive controls. The plates then were incubated at 30°C for 24 h before evaluation of their linear inhibitory zone.

Qualitative evaluation of digestive enzyme production

Starch agar, Tributyrin agar and Skim milk agar were the selective media for assessment of producing of amylase, lipase and protease. Enzyme activity will be revealed after 24-72 h of incubation by producing a transparent zone around colonies.

Statistical analysis

The possible difference between, bacterial plate count of different replicates, linear and diameter of Inhibitory zone produced by different candidate probiotic against pathogenic *Vibrios*, physicochemical differences between water from rearing tanks, and probable dissimilarity between shrimp weight of different tanks were assessed using one way analysis of variance, ANOVA. All statistical analysis was carried out using statistical software (Minitab 15, USA).

Results

Water quality and body weight of shrimp

The possible difference among water quality (physicochemical conditions) of shrimp tanks was analyzed. There was no significant difference between temperature, pH and salinity of different rearing tanks. Those were within suitable range for shrimp culture, 29±0.7°C, 7.9±0.1, 25±1 ‰, respectively. No mass mortality or disease was observed within rearing period. The resultant statistical analysis between juvenile shrimp body weight, 1.37±0.14 g of different larval tanks was not exhibited any significant difference ($P<0.05$), as well.

Bacterial count

Total aerobic heterotrophic bacteria, Total *Vibrio* and *Aeromonas*, Total *Enterobacteriaceae*, and total *Pseudomonas* of shrimp rearing water, sediment and different body parts including digestive system, muscles and body surface were counted using different selective media as demonstrated in table 1 and figure 1. Bacterial counts of different rearing tanks did not exhibited any significant difference ($P<0.05$).

Table 1: Bacterial count on different selective media (mean ± SD, n=3), from sediments, cultural water, shrimp body surface (CFU x 10^5/ ml), digestive system and muscles (CFU x 10^5/g) of *Penaeus monodon*

Media	Sediment	Cultural water	S.B.S.	D. S.	Muscle
TSA	530000± 50000	0.29±0.03	0.3±0.02	11±0.3	0.061±0.008
MacConkey A	-	-	-	-	-
TCBS A	12.87±0.777	0.121±0.0035	0.0117±0.0035	7.51±0.193	0.014±0.0038
PIA	2.07±0.351	0.012±0.0036	0.0953±0.0055	0.252±0.0125	0.034±0.004

TSA; Tryptone soy agar, MacConkey A; MacConkey agar, TCBS A; Thiosulfate citrate bile salt agar, PIA; *Pseudomonas* isolating agar, S.B.S.; Shrimp body surface, D.S.; Digestive System.

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According to morphological studies and conventional biochemical tests on total 118 isolates from digestive system, muscles, body surface, sediment and shrimp rearing water of juvenile *P. monodon* which was confirmed via Biolog GN and GP microplate (Olsson et al., 2004), 7 different genera were identified. Those were *Vibrio*, *Shewanella*, *Burkholderia*, *Clavibacter*, *Staphylococcus*, *Brevibacterium*, and *Corynebacterium*. While one group remained unidentified. The most frequent isolated bacteria from different body parts, sediment and rearing water were Gram negative (72%).

Antagonistic effect of candidate probiotic

Antagonistic properties of 118 isolate from juvenile black tiger shrimp were examined against shrimp pathogens, *V. parahaemolyticus*, *V. alginolyticus* and *V. harveyi*. Inhibitory ability of all 118 isolates from different body parts, sediment and shrimp rearing water against 3 shrimp pathogenic bacteria, *V. parahaemolyticus*, *V. alginolyticus* and *V. harveyi* were evaluated using Well diffusion plate technique. Table 2 and Figure 3 exhibited the diameter of inhibitory zone produced by 3 most antagonistic isolate against 3 shrimp pathogens. The antagonistic effect of 3 candidate probiotics were significantly different for each pathogenic shrimp *Vibrio*, The largest inhibitory zones against mentioned shrimp pathogens were produced by *Shewanella algae* followed by *Burkholderia glumae* and *Bravibacterium linens*.

Cross streak technique was the preferable method for further study on isolates that previously were demonstrated proper antagonistic ability against 3 cited shrimp pathogens. The tested isolates were fairly indicated identical inhibitory effect against mentioned shrimp pathogens. Three candidate probiotics revealed considerable antagonistic different against each pathogenic shrimp *Vibrio*. *S. algae* was generated the largest linear inhibitory zone against all 3 shrimp pathogens, the middle antagonistic power was produced by *B. glumae* and the weakest was made by *B. linens* (Table 3 and Figure 4).

Table 2: Diameters of inhibitory zone produced by three candidate probiotics against three shrimp *Vibrio* pathogens using well diffusion method

Vibrio pathogens	-VE Con.	*B. glumae*	*B. linens*	*S. algae*	+VE Con.
V. parahamolyticus	G	10.3	8.4	13.0	23
V. alginolyticus	G	10.0	9.0	13.5	23
V. harveyi	G	10.9	9.6	16.0	22

+VE Con.: positive Control, -VE Con.: Negative control; G: Grow

Table 3: Linear inhibitory zone of three candidate probiotics against Three shrimp *Vibrio* pathogens using cross streak method

Candidate probiotics	-VE Con.	+VE Con.	*V. parahamolyticus*	*V. alginolyticus*	*V. harveyi*
B. glumae	NG	G	14.1	21.2	40.4
B. linens	NG	G	G	12.4	25.5
S. algae	NG	G	22.0	27.3	43. 5

+VE Con.: positive Control; -VE Con.:Negative control; G: grow; NG: Not grew

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Digestive enzyme production

The candidate probiotic, *S. algae*, which was grown on Starch agar, Skim milk agar and Tributyrin agar exhibited ability to produce protease and lipase by consuming of protein and lipid of the media, while it was not able to utilize starch (Figure 5).

Discussion

Bacterial count

Enumeration of bacteria associated with environment and body parts of hatchery reared juvenile *P. monodon* were performed. Total aerobic heterotrophic bacteria count or total plate count of shrimp rearing water on TSA was 2.9×10^4 cfu/ml. There are quite large amount of information about total plate count of shrimp rearing water for different shrimp species at different stages under rearing conditions. Wang and his team (2005b) were published their studies on total bacterial count of new and 3 years old grow out pond which were under cultivation of *L. vannamei*. Their finding revealed that total bacterial count of recently constructed pond was 1.11×10^6 cfu/ml, while it was 6.25×10^6 cfu/ml for 3 years old pound. Sung et al. (2001) put out their finding on total bacterial count of 3 *P. monodon* grow out ponds which were in a range of 2×10^3 to 3×10^6 cfu/ml. Total plate count of shrimp rearing water in a number of *P. monodon* hatcheries in India was reported in ranges of 10^2 to 10^4cfu/ml and 10^4 to 10^7cfu/ml by Otta (2001). Yasuda (1980) exhibited that total plate count of rearing water of juvenile *P. japonicus* was lower than 10^4cfu/ml. Total bacterial count of fresh water shrimp, *Macrobrachium rosenbergii*, was analyzed and published by Kennedy (2006) which was ranged from 20×10^3 to 3.00×10^5 cfu/ml. Vaseeharan and Ramasamy (2003b) were broadcasted the mean value of total plate count of shrimp rearing tanks of 9 different *P. monodon* in India, which was $3.08 \pm 0.28 \times 10^5$cfu/ml. Although fairly wide ranges of total plate counts for rearing water of different shrimp genus and species in variety of growth stages including grow out pond and hatcheries were issued. But finding of total bacterial count from the current study was in quite agreement of the previous reports. This variability in the total plate counts of shrimp rearing water within and between different reports can be due to different management including feeding and water management, cultural system (traditional, extensive, semi intensive, intensive, and super intensive), source of water, water temperature, light regime, aeration system, or even stressful condition. Since bacterial population size influenced by presence of organic materials in water, which is directly affected with feed and water management and cultural system. In addition, water temperature as a critical factor should be considered for the cited differences. Presence of members of *Enterobacteriaceae* in sediments, rearing water and body parts of shrimp reared in grow out ponds of India was documented by Bhaskar et al. (1995), which was totally in contrast with finding of this study. There was no bacterial growth on MacConkey agar that demonstrated deficient of coliforms in different body parts, sediment, and rearing water of juvenile *p. monodon* in current study. This

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may be owed to different water sources or fertilizers used for water maturing.

The highest incident of *Vibrio* like microorganism, *pseudomonas* like microorganism and total bacteria was in sediment and digestive system of *P. monodon*, respectively. While those were minimum in case of body surface and shrimp rearing water (Table 1, Figures 1 and 2).

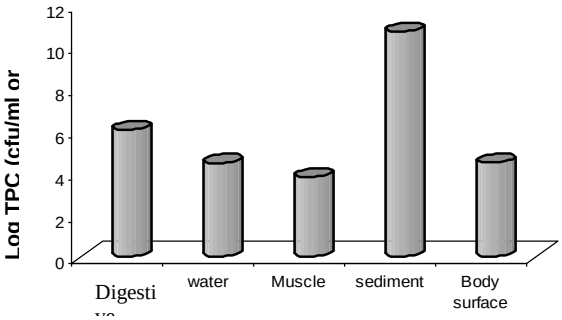

Figure 1:. Log Transferred TPC of Shrimp Different Body Parts, Sediment and Shrimp Rearing Water

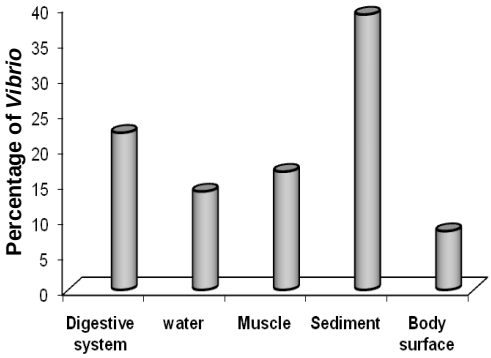

Figure 2: Comparison of *Vibrio* spp. Associated with Different Body Parts of *Penaeus monodon* and its Rearing Environment

Bacteria flora

Bacterial flora of hatchery reared juvenile *P. monodon* was mostly consisted Gram negative bacteria which were dominated by Vibrios. This result was exhibited good harmony with finding of Hamed (1993) in egg, larvae and post larvae of *P. indicus*. Studies of different researchers on bacterial flora of hatchery reared *Macrobrachium rosenbergii* revealed comparable results (Anderson et al., 1989; Phatarpekar et al., 2002; Kennedy *et al*., 2006). Yasuda (1980) was documented indistinguishable information about microflora which was associated with wild *P. japonicus*. All above findings are in parallel with the fact that bacteria flora of aquatic organisms are influenced by the microbial flora of their adjacent environment, which is dominated with Gram negative bacteria specially *Vibrio* spp. (Brisou et al., 1965).

Antagonistic effect of candidate probiotic

The isolated bacteria flora of different body parts and adjacent environment were subjected to two antagonistic tests, Well diffusion plate and Cross streak, against 3 shrimp pathogenic *Vibrios, V. parahaemolyticus, V. alginolyticus, and V. harveyi*.

Although the technique used for this study was not exactly identical with what Chythanya et al. (2002) utilized in their Well plate technique for assessing of a candidate probiotic, *Pseudomonas* I-2,

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against *V. harveyi*, but the obtained results are relatively alike. Their reported inhibitory zone was 17 mm for *Pseudomonas* I-2 which is relatively similar to inhibitory zone produced by *S. algae*, regardless the medium used in that study was TSA which is not the recommended media for *in vitro* antagonistic study and concentration of pathogens in liquid medium was not standardized. Utilization of different media for *in vitro* antagonistic test can be the main source of different due to their variable diffusion abilities of media as carrier for antagonistic substances; This is why MHA is the recommended medium for *in vitro* antagonistic tests. Furthermore, regardless to the differences of antibacterial susceptibilities of the pathogen strains used in these studies different concentration of pathogenic bacteria also revealed verity of inhibitory diameters when utilizing same antagonistic agent. The thickness of the media in bacterial plates may be considered as a source of bias, since the thicker media reduce the diffusion while the thinner media help better diffusion resulting in larger inhibitory zone. The inhibitory zone of candidate probiotics against pathogenic *Vibrios* in compare with the positive control, a synthetic commercial wide range antibiotic, seems to be quite acceptable as indicated in Table 2 and Figure 3. *S. algae* produced the best antagonistic effect against all pathogenic *Vibrio*s but its largest inhibitory zone was against *V. harveyi*. Based on antagonistic effect all candidates probiotics were revealed reasonable inhibitory ability against shrimp pathogens, but the best candidate probiotic bacterium was *S. algae*.

Chythanya et al. (2002) were documented relatively same result after assessment of antagonistic properties of *Pseudomonas* I-2 against *V. harveyi*, via cross streak method. Whilst several dissimilarity were occurred within using same technique such as, using different cultural medium, different pathogen strains, and standardization of pathogens concentration.

All 3 tested candidates probiotics are members of genus that previously known as probiotics; *Burkholderia ambifaria* in agriculture was considered as a biocontrol agent (Li et al., 2002), Utilization of *Bravibacterium* in water degradation of jellyfish was documented (Mimura and Nagata, 2001), and probiotic ability of 2 strains of genus *Shewanella* in marine aquaculture was well established (de la Banda et al., 2010). Among 118 bacteria which were isolated from different body parts, water and sediments, *Shewanella algae* exhibited highest antagonistic effect against 3 shrimp pathogenic *Vibrio*s.

Many researchers has been documented different substances such as protease, lysozyme, hydrogen peroxide, and bacteriocines that secrete form some bacteria and produce inhibitory effect on the growth of other bacteria (Fuller, 1987; Gatesoupe, 1999; Gomez-Gil et al., 2000; Irianto and Austin, 2002; Balcázar et al., 2006). Bacteriocines generated form lactic acid bacteria has been attracted huge attention due to viewpoint of consumption as natural preservatives in food (Cleveland et al., 2001). However, Brandelli Motta

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and Brandelli (2008) were put out that most of the bacterial species are able to produce antimicrobial peptides called bacteriocins, they added that within a certain species numerous different types of bacteriocines may be generated.

The antagonistic effect of the *S. algae* against mentioned Shrimp *Vibrio* pathogens may be caused by a bacteriocin like substances which has to be revealed by further study. Nevertheless, Candidate probiotics can be selected based on production of inhibitory compounds like bacteriocines, siderophores or when in competition for nutrients (Dopazo, 1988).

Digestive enzyme production

Contribution of microorganisms in digestion processes of bivalves by producing of extracellular enzyme such as, proteases, lipase and necessary growth factors is documented (Prieur, 1990). Similar observations have been reported for the microbial flora of adult shrimp, *Penaeus chinensis* (Wang et al., 2000; Wang, 2007). Ability of *S. algae* to produce digestive enzymes like, protease and lipase may contribute and help shrimp to have higher digesting efficiency, lower FCR and higher growth.

Colonization of candidate probiotic in the digestive system of the host

Shewanella is the second dominant *genus* (23.7 %) which was isolated from *P. monodon* body parts and its rearing environment. The predominant genus was *Vibrio* consisting of 30.5 % of total identified bacterial flora. Incidence of *Burkholderia, Clavibacter, Staphylococcus, Corynebacterium* and *Bravibacterium* are 17.8, 9.3, 8.5, 5.9 and 2.5, respectively. Among identified Genera, *Shewanella* is the most frequent genus in the digestive system of the *P. monodon* (Fig. 6) consisted of 9.3% of total isolated bacteria, while its abundance in sediment and rearing water is quite lower when comparing with genus *Vibrio* which is predominant bacterial flora of *P. monodon* in the current study and exhibiting higher incidence in sediment. This may imply that digestive system of *P. monodon* has good condition for colonization of *Shewanella*.

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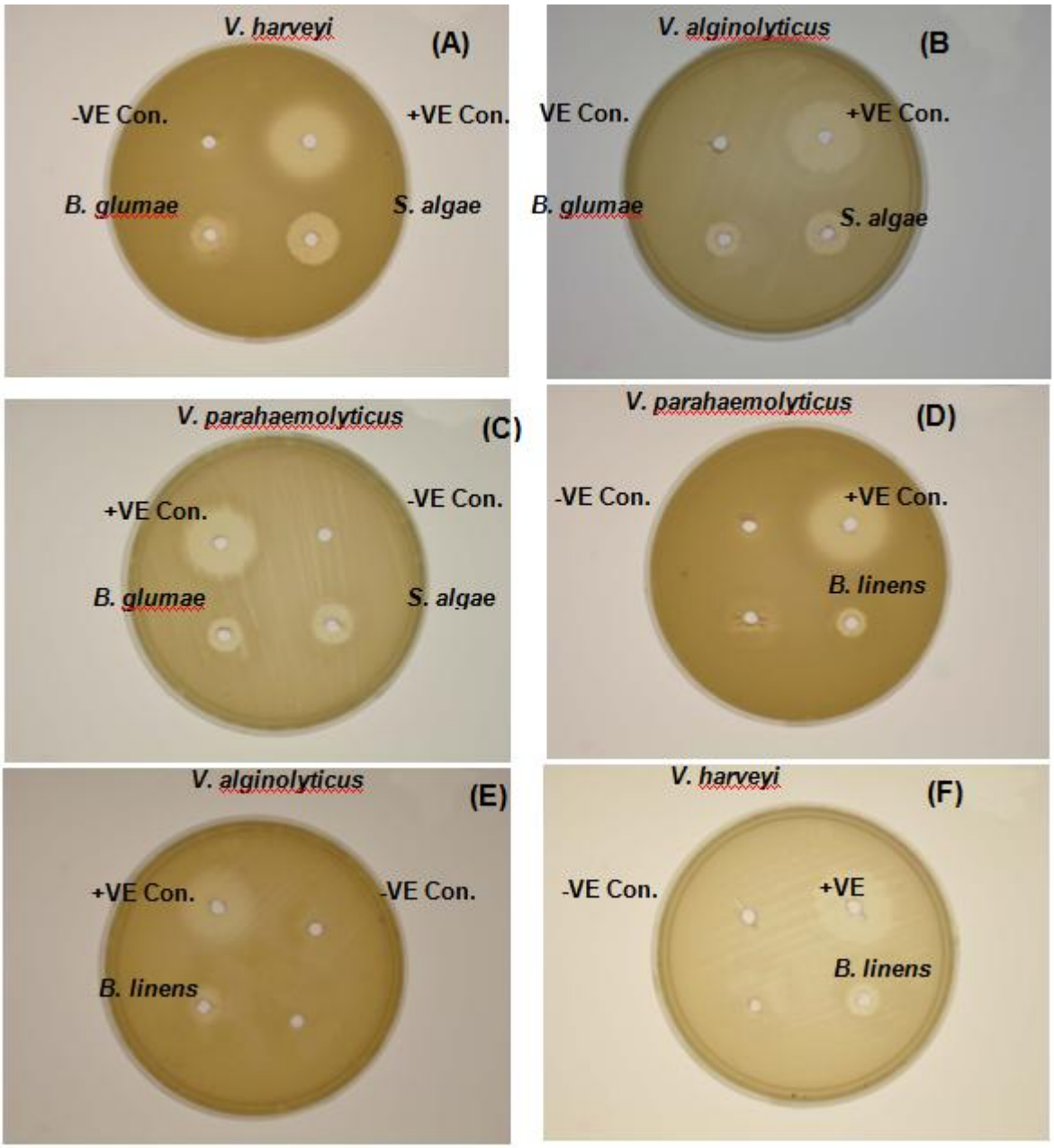

Figure 3: Well Diffusion Plate Antagonistic Effect of Three Candidate Probiotics, *S. algae*, *B.glumae* and *B.linens* Against Three *Vibrio* Pathogens, *V. parahaemolyticus*, *V. alginolyticus* and *V. harveyi*. –VE & +VE Con.: Negative and Positive Controls, unlabeled wells were not used in test

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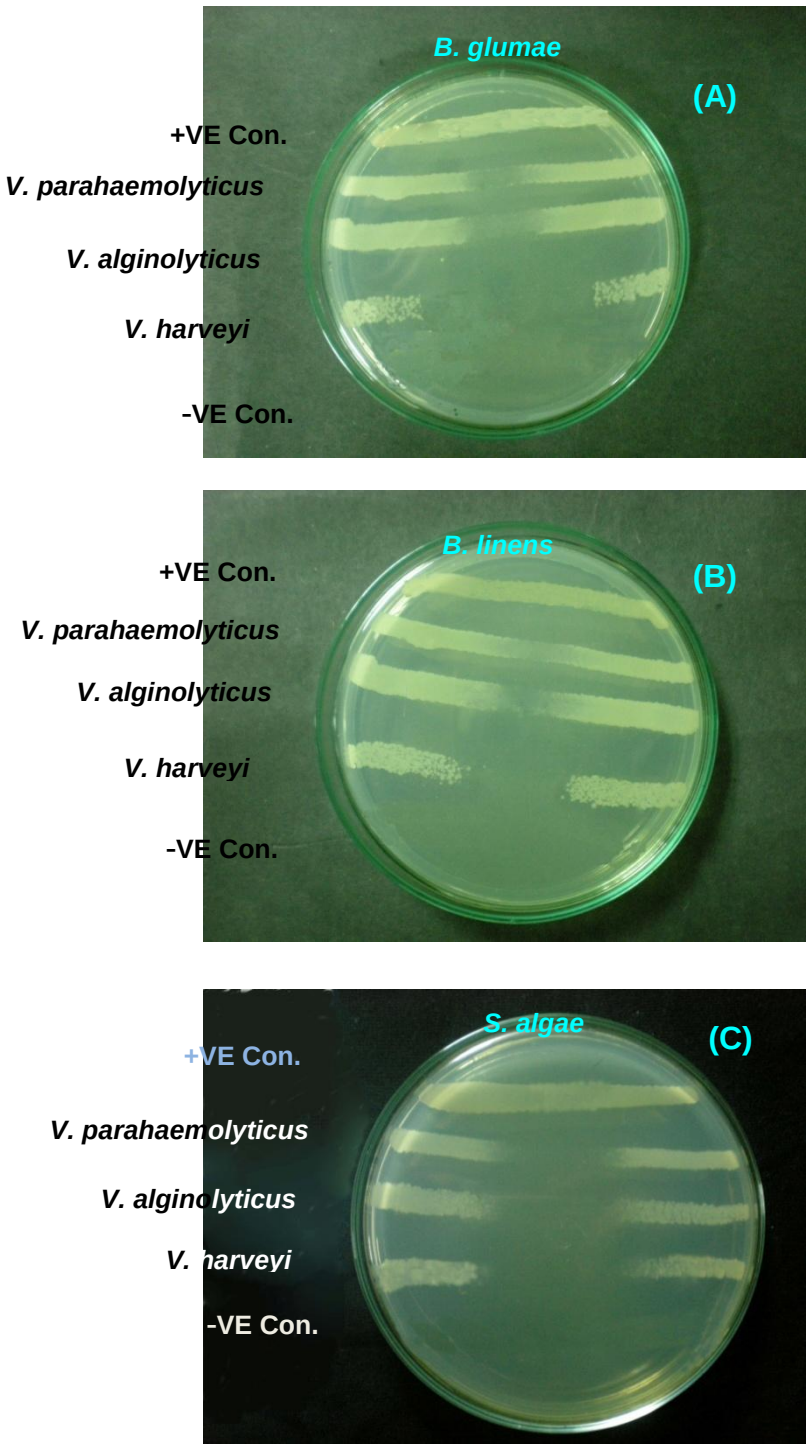

Figure 4: Linear Inhibitory Zone Produced by Candidate Probiotics, *S. algae*, *B. linens* and *B. glumae*, Against *Vibrio* Pathogens, *V. harveyi* *V. parahaemolyticus* and *V. alginolyticus* Using Cross Streak Method. –VE & +VE Con.: Negative and

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Figure 5: Protease and Lipase activity of *S. algae* on SMA plate (A) and TBA Plate (B). SMA: Skim milk agar, TBA: Tributyrin agar

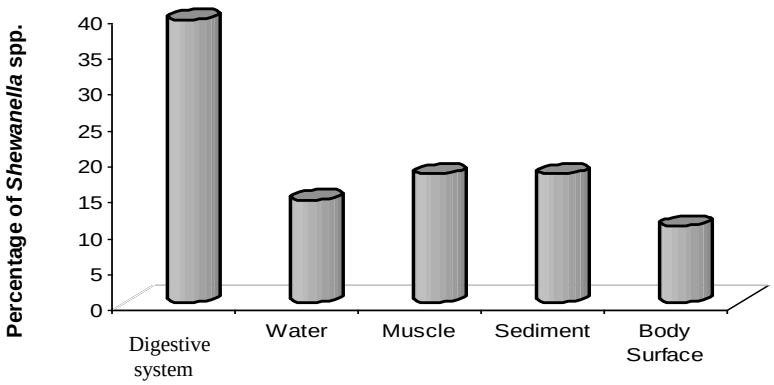

Figure 6: Comparison of *Shewanella* spp. Associated with dfferent body parts of *Penaeus monodon* and Its Rearing Environment (source: Shakibazadeh et al., 2009)

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