JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Nov 2015. Vol. **9**(Spl. Edn. 2), p. 407-420

Screening of Bacterial Pathogens Attached to Invertebrate Bodies and Biocontrol of Pathogenic *Aermonas hydrophila* in *Marsupenaeus japonicus* Culture

Sahar W.M. Hassan[1], Hanan Abd-Elnaby[1] and Ragia M. Moussa[2]*

[1]Microbiology Laboratory, Environmental Division,
National Institute of Oceanography and Fisheries, Alexandria, Egypt.
[2]Invertebrate Aquaculture Laboratory, Aquaculture Division,
National Institute of Oceanography and Fisheries, Alexandria, Egypt.

(Received: 30 June 2015; accepted: 15 September 2015)

Microbes play crucial role in aquaculture systems. In the present study, screening of the bacterial communities of *Aeromonas* sp., *Vibrio* sp., *Staphylococcus* sp., *E. coli* sp. and *Salmonella* sp. associated with shrimp, bivalves and sea cucumber revealed that *Aeromonas* sp. was the dominant pathogen in shrimp representing 6.33% and 11.25% of the total heterotrophic bacteria associated with shrimp surface and body, respectively. Antibacterial activity of different extracts of the invertebrate bodies and chitosan in addition to eleven attached bacterial isolates were tested. The marine bacterial isolate which was identified as *Vibrio alginolyticus* by using 16S rDNA sequence analysis, was superior in its antibacterial activity, recording the highest activity unit of 10.24 ± 3.15 AU against *A. hydrophila.* The impact of probiotic *V. alginolyticus* S10 in improving the water quality of *A. hydrophila* infected *Marsupenaeus japonicus* culture was investigated. The histopathological study of *M. japonicus* tissues showed limited tissue damages observed in treatments using probiotic bacteria. Three stages of infection have been defined in infected hepatopancreas according to vacuolation, and hemolytic aggregation while intensity of infections in muscle and heart tissues was determined according to bacterial doses and hemolytic aggregation.

Key word: *Vibrio alginolyticus* S10**,** *Aeromonas* sp., Antibacterial, Probiotic, *Marsupenaeus japonicus***,** Histopathology**.**

The shrimp farming becames a highly competitive and profitable farming practice in many countries worldwide[1]. In Egypt, *Marsupenaeus japonicus* composes much of the prawn catch off the Egyptian Mediterranean coast and in the Nile delta lagoons. It is considered one of the most economically important members of the family Penaeidae[2]. Globally, its greatest importance in aquaculture has been arisen since 2003 as the annual catch value has been exceeded $200 million[3]. On the other hand, the observed rapid expansion programs of shrimp culture worldwide are hindered by diseases affecting production; and outbreaks of diseases causing major problems in many countries[4].

Diseases have been considered as one of the major constraints to shrimp farming development[1]. In the past decades, the shrimp farming has serious problems due to some bacterial, fungal, viral and protozoal diseases[5,6] leading to huge economic losses as a result of sever mortality and production rate decline[6,7]. Among the significant disease agents of shrimp culture, bacterial diseases are the most popular in terms of severity and impact[7,8]. The most common bacterial diseases in shrimp aquaculture were known as Septicemia, and Vibriosis, which were caused by *Aeromonas* sp.[9], and *Vibrio* sp.[6,10], respectively, and indirectly affected the shrimps' health through

* To whom all correspondence should be addressed.
E-mail: ragiamoussa@yahoo.com.au

worsen the water quality[11]. With regard to aeromonads, they are the major pathogens in the fisheries sector inflicting serious damage in pond and aquarium cultures[12,13]. Therefore, perpetual need for new chemotherapeutants is needed to combat new diseases and drug-resistant pathogens that are becoming a significant threat not only for aquaculture but also for the public health[13,14].

In aquaculture, treatment with disinfectants and antimicrobial drugs is a common approach to control diseases, however, it has limited success in the prevention or cure of aquatic diseases due to increasing virulence of pathogens[15]. However, the use of probiotic biocontrol agents instead of antibiotics becomes from the major concerns to improve disease resistance, water quality and/or growth in aquatic systems[16-18]. Probiotic microorganisms may release chemical substances such as antibiotics, bacteriocins, siderophores, lysozymes and proteases that affect on other microbial populations[14]; they do so by inhibition of the colonization of potential pathogens, alteration of the microbial metabolism, alteration of pH values by organic acids production and/or stimulation of the host immunity. In addition, probiotics may stimulate appetite and improve nutrition by the production of vitamins or detoxification of compounds in the diet[18].

As marine biodiversity richness assumes a great opportunity for the discovery of new bioactive compounds, approximately 6,500 bioactive compounds have been isolated from the marine organisms[19]. Within the scope of the bioactive compounds, chitin and chitosan are unique and typical marine polysaccharides waiting for future development and have been attracted the interest of many researchers from various disciplines. Chitin is the second most important natural polymer in the world. It lacks of toxicity and allergenicity, its biocompatibility, biodegradability and bioactivity make it a very attractive substance for diverse applications as a biomaterial in the pharmaceutical and medical fields[20]. Moreover, it was stated that antibacterial activity of chitosan is effective in inhibiting bacterial growth[21]. The antimicrobial properties of chitosan depend on its molecular weight and the type of bacterium.

The present study was proposed to (i) address the occurrence of associated bacterial pathogens in some invertebrates, (ii) asses the potentiality of the attached bacteria and some invertebrates extracts as antibacterial agents, and (iii) investigate the elimination of *A. hydrophila* in *M. japonicus* culture by using the best isolated probiotic with detailed histopathological study of different tissue organs.

MATERIALS AND METHODS

Samples collection and adaptation

Sea cucumber, bivalves and shrimp were collected from trawlers in Abu Qir Harbor in West Alexandria, Egypt, during winter 2012. Fresh samples were packed in ice box and transferred to National Institute of Oceanography and Fisheries, Alexandria, Egypt for microbiological studies.

For investigation of probiotics impact on pathogenic bacteria in *M. japonicus* aquarium, live shrimp samples were caught from Abu Qir (1° 19' 19" N, 30° 3' 39" E) , Alexandria. Before examination, shrimp were maintained in fiberglass tanks and acclimated to laboratory conditions for a week. During the acclimation period, shrimp were fed twice daily with commercial pellet (40% protein content). Water of shrimp aquarium was changed daily in rate of 30%. During the study, water temperature, pH and dissolved oxygen were daily monitored as 25±1ºC, 8-8.5 and 5- 5.5 ppm, respectively.

Bacterial isolation and enumeration

One gram of each invertebrate sample (shrimp, bivalves and sea cucumber) was shaken in 9 ml sterile sea water for 30 min, to dissociate the adhered bacterial population. The upper layer was used as the initial dilution for bacteriological analysis. For estimation of total viable count (TVC), Decimal dilutions were prepared and cultivated on Zobell agar using pour plate method and incubated at 30 ºC for 24h. For detection and counting the thermotolerant coliforms (*E. coli*), the m-FC agar medium was used and the plates incubated at 44.5ºC for 24 h. Counting of *Aeromonas* sp. was carried out on m-*Aeromonas*-selective agar medium with ampicillin antibiotic supplement (SR O136). Mannitol salt agar medium was used for *Staphylococcus* sp. detection. *Vibrio* sp. was detected using Thiosulphate Citrate Bile Salt (TCBS) agar while *Salmonella* sp. was detected

on *Shigella–Salmonella* (SS) agar. All plates incubated at 37 ºC for 48h[22]. Three replicates for each sample were used and the final counts were estimated as colony forming units (mean ± SD CFU g^{-1}).

Bioactive compounds

Bacterial supernatant

Eleven different isolates (S1-S11) of the bacteria associated with shrimp, sea cucumber and bivalves were selected and tested for their antimicrobial activity against different Gram positive and Gram negative bacteria. The selected bacterial isolates were grown in marine nutrient broth at 30ºC for 24 h. The culture broth of each isolate was centrifuged at 10000 rpm for 15 minutes to remove bacterial cells. The inhibition of pathogenic bacteria by the cell free supernatant was tested by the agar well-cut diffusion method[23].

Extraction from different invertebrate bodies

The bioactive compounds from the whole body of different invertebrate (shrimp, bivalves or sea cucumber) samples were extracted as follow. Sample of each body (5 g) were homogenized and extracted with different solvents, 10 volumes (v/w) of methanol, acetone or hexane. After soaking for a week, the supernatant of each sample was collected by centrifugation at 10000 rpm for 15 minutes, then filtered through a 0.2 µm millipore filter and the sterile filtrates were used for the antimicrobial assay[24].

Extraction of chitosan from the crustacean shell wastes

Isolation of chitosan from shrimp shell wastes involves four traditional steps demineralization, deproteinization, decolorization, and deacetylation[25]. The antibacterial activity of chitosan was tested using well-cut diffusion technique.

Antimicrobial activity assay

Bacterial indicators

Different Gram negative and Gram positive bacteria including *Escherichia coli, Staphylococcus aureus* ATCC 6538, *Streptococcus faecalis, Bacillus subtilis, Aeromonas hydrophila, Pseudomonas aeruginosa* ATCC 8739, *Vibrio anguillarum and Vibrio fluvialis* were used as target strains for detecting the antagonistic properties. Various indicator bacteria were previously isolated from several marine sources used in previous studies by the aid of National Institute of Oceanography and Fisheries, Egypt.

The well-cut diffusion technique

The well-cut diffusion technique was used to test the ability of the cell free supernatant, the body extracts and chitosan to inhibit the growth of the indicator bacteria. Five-millimeter-diameter wells were punched in Zobell agar plates (using a sterile gel puncher) inoculated with bacterial pathogenic strains. 50 µl of each tested compound was added in each well. After incubation at 30ºC for 24h, The activity unit (AU), which indicates a positive result in the antagonistic action, was calculated according to the following equation: $AU= Y^2/X^2$. Where, Y is the radius of the clear zone around the zone and X is the radius of the well itself[26, 27]. Three replicates were used for each and the final values were estimated as mean ± SD.

Bacterial identification

DNA was isolated, purified using standard procedures[28] and the region of 16S rDNA was amplified using universal primers. Genotypic characterization was performed using 16S sequence analysis. Multiple alignments with sequences of most closely members and calculations of levels of sequence similarity were carried out using Blast program (http://www.ncbi.nlm.nih.gov/blast). Sequences of rRNA genes, for comparison, were obtained from the NCBI database. A phylogenetic tree was reconstructed by Bioedit software.

Probiotic application for elimination of *A. hydrophila* from *M. japonicus* culture

Preparation of the bacterial suspension'

Bacterial isolate (S10) which exhibiting the highest antibacterial activity against *A. hydrophila* was identified as *Vibrio alginolyticus* S10. *Vibrio alginolyticus* S10 was used as a probiotic in *M. japonicus* culture infected by *A. hydrophila*.

Vibrio alginolyticus was precultured in marine nutrient broth at 30 ºC on a rotary shaker incubator until the absorbance of the culture at A_{550}=1. The number of *Vibrio alginolyticus* S10 was estimated by preparing 10-fold serial dilutions then 0.1 ml from each dilution was inoculated on thiosulphate citrate bile agar plates. *A. hydrophila* was prepared as described before and enumerated on m-*Aeromonas*-selective agar plates.

Effect of the probiotic on elimination of *A. hydrophila* from *M. japonicus* culture

About seventy two *M. japonicus* (Bate, 188) specimens were used with average weight of 17.55±0.7 g, with no significant size differences among the treatments. Four treatments were infected with 10^4 CFU $ml^{-1}$of bacterial pathogen *A. hydrophila*, three of them (1, 2 and 3) were treated with probiotic (*Vibrio alginolyticus* S10) at the concentrations of 10^4, 10^5 and 10^6 CFU ml^{-1}, respectively, while the forth treatment was the control without probiotic.

The density of *Aeromonas* sp. associated with water was monitored daily during experimental period. Counting was applied by spreading 100 µl of shrimp culture water over m-*Aeromonas* agar medium with ampicillin selective supplement (SR O136), and incubating at 37°C for 24 h[22]. Any dead shrimp was daily recorded and removed.

Histopathological study

Tissues samples from *M. japonicus* hepatopancreas, heart, and muscle were examined ultrastructurally in freshly caught samples, dead samples of unexplained mortality and in fresh survived samples at the end of experimental period. The shrimp tissues were fixed in Bouin fixative for 48 h and transferred to 70% ethyl alcohol. After processing and hydration of tissues, wax impregnation was done. The paraffin wax embedded samples were sectioned, mounted on slides and stained with Myer-bennet haematoxylin and pheloxin/eosin[29].

Statistical analysis

Data analysis was performed with the software package Microsoft Excel, version 2003. Data from experimental infection analyzed by the t-test analysis ($p < 0.05$)[30].

RESULTS AND DISCUSSION

Bacteria are considered among the economically significant disease agents of shrimp culture[8] resulting in poor water quality and bad management[31]. Bacterial disease of shrimp culture is the driving factor of ecological balance breakdown within the culture system. Many of these bacteria are normal inhabitants of the marine environment. Therefore, the present study screened the occurrence of different bacterial communities associated with edible invertebrates.

Community composition

There are two groups of bacteria relevant to public health that contaminate marine products: bacteria naturally present in the environment such as *Aeromonas hydrophila, Clostridium botulinum, Vibrio parahaemolyticus, V. cholerae, V. vulnificus* and *Listeria monocytogenes*, and Enterobacteriaceae such as *Salmonella sp., Shigella sp.* and *Escherichia coli*[32].

Bacterial flora attached to invertebrate surface

The heterotrophs count attached to the surface of invertebrate samples (shrimp, bivalves and sea cucumber) (Fig. 1 & Table 1) showed that, the total viable count ranged from $9.0x10^3 \pm 1.0x10^3$ CFU g^{-1} in shrimp to $3.6x10^4 \pm 4.0x10^3$ CFU g^{-1} in sea cucumber Almost the surface of the invertebrate samples harbored high counts of bacteria. invertebrates surface are more nutritious than inanimate material and a large number of marine bacteria could live on it acquiring necessary nutrition such as vitamins, polysaccharides and fatty acids from their hosts[33].

In the present study, screening of the attached *Aeromonas* sp. *Vibrio* sp., *Staphylococcus* sp., *E. coli* sp. and *Salmonella* sp. to the surface of invertebrate samples such as shrimp, bivalves and sea cucumber showed varied counts of pathogenic bacteria. The highest observed counts of pathogenic bacteria and occurrence percentages were for *Aeromonas sp*. It ranged between $5.7x10^2 \pm 9.4x10^1$ CFU g^{-1} in shrimp to $1.7x10^3 \pm 3.2x10^2$ CFU g^{-1} in sea cucumber, with occurrence percentages 6.33% and 4.72%, respectively. As wise, it was reported that *A. hydrophila* is a major pathogen of aquatic and terresterial organisms[5].

Additionally, the highest counts ($1.2x10^3 \pm 4.0x10^2$ CFU g^{-1}) of *Staphylococcus* sp. was harbored in sea cucumber with occurrence percentage of 3.33% while shrimp had the lowest counts ($3.2x10^2 \pm 4.3x10^1$ CFU g^{-1}) with occurrence percentage of 3.56%. With regard to the *Vibrio* sp., it was reported that seafood from marine and estuarine environments is impossible to be free from it[34]. In the present study, the *Vibrio* sp., counts ranged from $2.0x10^2 \pm 6.0 x10^1$ in bivalve to $1.0x10^2 \pm 1.7x10^1$ CFU g^{-1} in shrimp representing 8.33% and 2.22% of the total viable counts, respectively.

Salmonella sp. exhibited the lowest occurrence as compared to the other pathogens and was detected only in shrimp ($1.5x10^2 \pm 6.0x10^1$ CFU g^{-1}) with 1.67% occurrence percentage. The

Table 1. The occurrence percentages (%) of the different pathogens attached to the surface of shrimp, bivalves and sea cucumber with respect to the total viable count

	TVC (CFU/g^{-1})	Occurrence %				
		Aeromonas sp.	*Staphylococcus* sp.	*Vibrio* sp.	*E. coli*	*Salmonella* sp.
Shrimp	9.0 x10^3 ±1.0 X10^3	6.33	3.56	2.22	4.44	1.67
Bivalve	1.2 x10^4 ± 2.0 X10^3	7.75	4.33	8.33	4.00	0.00
Sea cucumber	3.6 x10^4 ± 4.0 X10^3	4.72	3.33	0.83	0.11	0.00

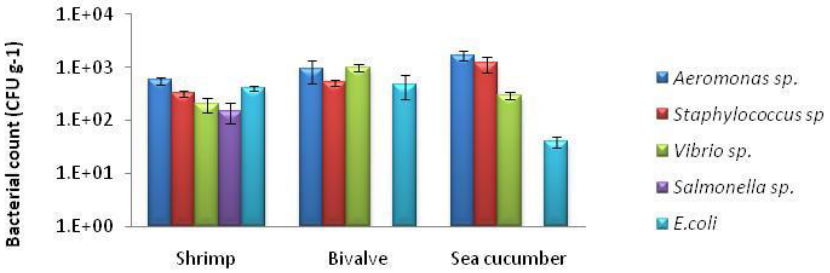

Fig. 1. The counts of some pathogens attached to the surface of shrimp, bivalves and sea cucumber

maximum counts of *E. coli* (4.8×10^2 ± 2.2×10^1 CFU g^{-1}) was detected in bivalve with occurrence percentage of 4% followed by shrimp (4.0×10^2 ± 4.5×10^1 CFU g^{-1}) with occurrence percentage of 4.44% while sea cucumber exhibited the lowest counts (4.0×10^1 ± 1.0×10^1 CFU g^{-1}) with 0.11% occurrence percentage (Fig. 1 and Table 1). In this context, *Aeromonas sp*. was the highest count harboring the shrimp surface among other pathogenic bacterial groups.

Bacterial flora within body flesh

The presence of pathogenic bacteria in marine invertebrate such as shrimp causes several waterborne infections in humans that are worldwide concerning issues[35]. Estimation of the same pathogenic groups in the body flesh of the tested invertebrate samples was carried out (Figure 2 and Table 2). The heterotrophs count ranged from 2.0 ×10^4 ± 8.0 ×10^3 CFU g^{-1} in bivalves to 5.0 ×10^4 ±1.2 ×10^4 CFU g^{-1} in sea cucumber. Counts of *Aeromonas sp.* ranged from 5.4 ×10^3 ± 1.1×10^1 CFU g^{-1} in shrimp to 2.8×10^3 ± 6.3×10^2 CFUg^{-1} in bivalve with occurrence percentages of 11.25% to14.00 %, respectively. It was reported that *Aeromonas* and *Vibrio sp*. were found in the shrimp and were dominant among other bacterial species[35].

Regarding *Staphylococcus* sp., the highest count 5.7×10^3 ± 9.3×10^2 CFU g^{-1} was recorded in bivalve while the lowest count (2.7×10^3 ± 8.1×10^2 CFU g^{-1}) was noticed in the sea cucumber with occurrence percentages of 28.5% and 5.4%, respectively. Generally, reports on isolation of staphylococci from shellfish are limited but they are well recognized as causative agents of food poisoning and may constitute a serious risk to human health if present in consumed raw bivalve molluscs[36]. Contrary to the present finding, it was reported the dominance of other bacterial species such as *Aeromonas*, *Plesiomonas*, *Photobacterium*, *Pseudoalteromonas*, *Pseudomonas* and *Vibrio*[37].

In addition, the present study revealed the absence of *Salmonella* sp. in bivalve samples as reported by Ripabelli *et al*. (1999) [38], who analyzed 62 mollusk samples from the Adriatic Sea and searching for the presence of verotoxigenic *Vibrio*, and *E. coli* and detected no *Salmonella* strains.

Sea cucumber exhibited the highest counts (3.3×10^3 ± 6.7×10^2 CFU g^{-1}) of *Vibrio* sp. with occurrence percentage of (6.72%) while the lowest count (1.2×10^2 ± 3.0×10^1 CFU g^{-1}) was for shrimp with occurrence percentage of 0.25%.

Absence of *E. coli* and *Salmonella* counts was observed in the tested samples except for shrimp which harbored 1.0x10^3 ± 2.0x10^2 CFU g^{-1} of

E. coli with 2.08% and bivalves which contained $1.0x10^2 \pm 3.9x10^1$ CFU g^{-1} of *Salmonella* with 0.5 % occurrence percentage.

Antimicrobial activity

The bacterial supernatant

Competition amongst microbes for space and nutrients in the marine environment is a powerful selective force which has led to the evolution of a variety of effective strategies for colonizing and growing on surfaces. Thus it is expected that the antibiotic-producing bacteria associated with some particular hosts are proportionally higher than others[39]. Production of antimicrobial agents by bacteria associated to invertebrates was previously documented in different studies[40]. It was reported that over 400 strains of surface-associated bacteria from various species of seaweed and invertebrate from Scoogttish coastal waters were isolated and 35% of them shown to produce antimicrobial compounds[39]. This is a much higher proportion than free living marine isolates. It was also stated that the proportion of the active marine bacteria associated with some invertebrates was higher than those associated with other organisms and the antagonistic percentage against different indicator pathogenic bacteria reached up to 20%. In the present study, the antagonistic percentage of associated bacteria against the indicator pathogenic bacteria reached more than the previously reported one. The isolated bacteria showed varied ranges of antimicrobial spectra, where 36.4% of the isolates exhibited antibacterial activity against *S. faecalis* followed by antagonistic percentage of 27.3% against *V. fluvialis*, *A. hydrophila* and *E.coli* , while 18.18% of the isolates showed antagonistic activity against *S. aureus* and *P. aeruginosa*, however the lowest percentage of the isolates (9%) was active against *V. anguillarum*. On the other hand there was absence of antagonistic action against *B. subtilis*. Bacterial isolate S10 recorded the highest activity unit (10.24 ± 3.15 AU) against *A. hydrophila* and exhibited broad spectra of antimicrobial activity against *P. aeruginosa, S. aureus and E. coli* with activity units of 5.76± 1.74 AU, 5.76 ± 2.78 AU, and 9.00 ± 1.76 AU, respectively (Table 3). It can be concluded that the highest antagonistic activity (36.4%) was against Gram positive bacteria which agree with other studies[40]. This phenomenon seemed owing to the multidrug efflux systems lay in the Gram negative bacteria. Lower outer membrane permeability was also expected to contribute greatly to intrinsic resistance of Gram negative bacteria to a wide range of antibiotics[41].

The body extracts

The innate defense mechanisms of aquatic invertebrates against pathogenic

Table 2. The occurrence percentages (%) of the different pathogens in the body of shrimp, bivalves and sea cucumber with respect to the total viable count

	TVC (CFU/g^{-1})	Occurrence %				
		Aeromonas sp.	*Staphylococcus* sp.	*Vibrio* sp.	*E. coli*	*Salmonella* sp.
Shrimp	$4.8\ X10^4 \pm 1.7\ X10^4$	11.25	8.33	0.25	2.08	0.00
Bivalve	$2.0\ X10^4 \pm 8.0\ X10^3$	14.00	28.50	6.00	0.00	0.50
Sea cucumber	$5.0\ X10^4 \pm 1.2\ X10^4$	8.00	5.40	6.72	0.00	0.00

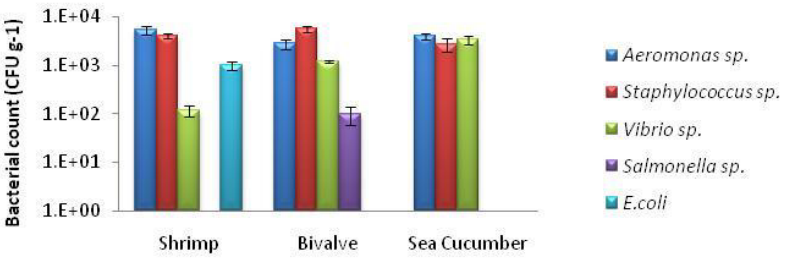

Fig. 2. The counts of some pathogens in the body of shrimp, bivalves and sea cucumber

organisms have made them prime candidates for extraction of microbicidal compounds[42]. In the present study, the antibacterial activity of the body extracts using methanol, acetone and hexane against the previously mentioned indicator pathogenic bacteria was tested. The highest antibacterial activity unit (7.84 ± 1.76 AU) was recorded for acetone extract of shrimp against *V. anguillarum* followed by methanolic extract of shrimp against *A. hydrophila* (5.76 ± 1.92 AU). Sea cucumber also exhibited antimicrobial activity represented in acetone extract against *V. fluvialis* (4.84± 1.41 AU) and hexane extract against *B. subtilis and A. hydrophila* with activity units 4.84 ± 1.84 AU and 4.84 ± 1.92 AU, respectively, while there was no activity for the other solvent extracts (Table 4).The difference in antibacterial activity found with extracts may be attributed to the difference in extracting capacity of solvents and extracted compound[43].

The extracted chitosan

In the present study, chitosan was effective only against Gram negative bacteria. (*V. anguillarum*) with 2.7±0.87 AU (Table 4), while it was reported that it has antimicrobial activity against both Gram positive bacteria (*S. aureus*) and Gram negative bacteria (*E. coli*)[44]. It was stated that the antimicrobial activity of chitosan is described to be associated with molecular weight degree, acetylation, concentration of chitosan and bacterial inoculum size[34]. It was showed that lower molecular weight chitosan is more effective against Gram- negative bacteria, while high molecular weight chitosan is effective against Gram-positive bacteria[45]. The present study concluded that the antibacterial activity of the associated bacterial isolate (S10) was more effective and exhibited broader spectra of activity against the tested Gram negative and Gram positive bacterial pathogens in comparison to the body extracts of the tested invertebrates and the chitosan. Thus, the most potent bacterial isolate (S10) was chosen to complete the study.

Molecular characterization of the potent bacterial isolate

In the present study, the DNA of the most effective marine bacterial isolate (S10) exhibited broad spectra of antimicrobial activity was extracted. The extracted 16S rRNA gene (approximately 1500 base pair) was amplified using the universal primers. The amplified DNA was partially sequenced. This sequence was compared with those which gave the highest homology using Blast search computer based program. The resulting data indicated that the isolate (S10) under study was identified as *Vibrio alginolyticus* S10. The obtained similarity was 98% with session number KP280001. The phylogenetic relationships of this experimental isolate and the closely related relatives were analyzed as shown in Figure 3. Antagonism is considered an important attribute of aquaculture probionts and is thus widely used as a selection criterion to select potential probiotic bacteria[14]. *V. alginolyticus* S10 was subsequently evaluated for its bacterial ability to out-compete a target organism in vitro, and was selected to use

Table 3. Antibacterial activity of the different bacterial isolates against some indicator bacterial pathogens

Bacterial isolates	Antibacterial activity unit (AU) of different bacterial isolates against the pathogens							
	E. coli	*S. aureus*	*S. faecalis*	*B. subtilis*	*A. hydrophila*	*P. aeruginosa*	*V. anguillarum*	*V. fluvialis*
S1	6.76± 1.73	-	-	-	-	-	-	9.00 ± 2.11
S2	-	-	-	-	-	-	-	6.76 ±1.75
S3	-	-	7.84 ±1.64	-	-	-	-	-
S4	-	-	6.76 ±2.16	-	-	-	-	-
S5	-	9.00 ± 1.20		-	-	-		-
S6	-	-	5.76 ±.95	-	-	-		-
S7	-	-	-	-	-	-	7.84 ± 2.86	-
S8	-	-	-	-	-	-	-	-
S9	-	-	-	-	6.76 ± 1.13	6.76 ± 1.98	-	4.84 ±1.64
S10	9.00 ±1.76	5.76 ±2.78	-	-	10.24 ± 3.15	5.76 ± 1.74	-	-
S11	7.84 ± 2.58	-	6.76 ±1.76	-	5.76 ± 2.13	-	-	-

Table 4. Antibacterial activity of the body extracts and chitosan against some indicator bacterial pathogens

Extract	Antibacterial activity unit (AU) of body extracts and chitosan against							
	E. coli	*S. aureus*	*S. faecalis*	*B. subtilis*	*A. hydrophila*	*P. aeruginosa*	*V. anguillarum*	*V. fluvialis*
Methanol extract of								
Shrimp	-	-	-	-	5.76 ± 1.92	-	-	-
Bivalve	-	-	-	-	-	-	-	-
Sea cucumber	-	-	-	-	-	-	-	-
Acetone extract of								
Shrimp	-	-	-	-	-	-	7.84 ± 1.76	-
Bivalve	-	-	-	-	-	-	-	-
Sea cucumber	-	-	-	-	-	-	-	4.84± 1.41
Hexane extract of								
Shrimp	-	-	-	-	-	-	-	-
Bivalve	-	-	-	-	-	-	-	-
Sea cucumber	-	-	-	4.84 ± 1.84	4.84 ± 1.92	-	-	-
Chitosan	-	-	-	-	-	-	2.7± 0.87	-

as probiotic in *M. japonicus* culture. There are several microbial strains used as probiotics in aquaculture systems. The common probiotics used in aquaculture belonging to genus *Lactobacillus*, *Bacillus*, *Vibrio*, *Enterococcus* are now used for oral bacterio-therapy in aquaculture[45].

Probiotic application for elimination of *A. hydrophila* from *M. japonicus* culture

Shrimp aquaculture, as well as other industries, constantly requires new techniques in order to increase production yield. Modern technologies and other sciences such as biotechnology and microbiology are important tools that could lead to a higher quality and greater quantity of products[46]. Probiotics, as 'bio-friendly agents' can be introduced into the culture environment to control and compete with pathogenic bacteria as well as to promote the growth of the cultured organisms. In addition, probiotics are nonpathogenic and nontoxic microorganisms without undesirable side-effects when administered to aquatic organisms[14,47].

Bacterial growth

The counts of *A. hydrophila* were estimated daily in *M. japonicus* culture water for seven successive days (Fig. 4). The growth of

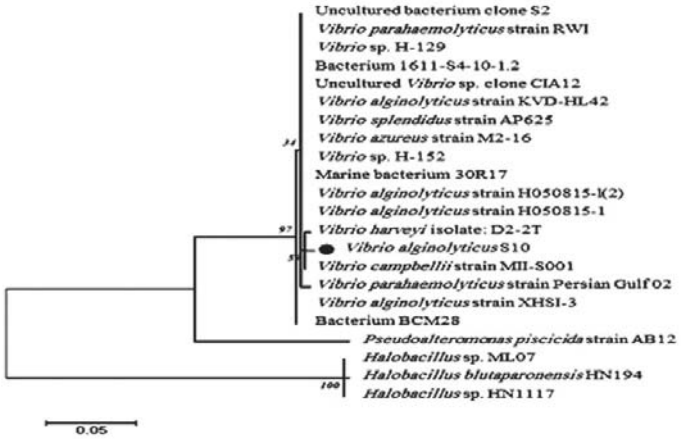

Fig. 3. Phylogenetic relationships among the representative experimental strain and the most closely related *Vibrio* species. The dendogram was generated using tree view program

pathogenic *A. hydrophila* was inhibited by *V. alginolyticus* S10 (Fig. 4). Lower concentrations of *V. alginolyticus* S10 culture inoculated at an initial concentration of 10^4 and 10^5 CFU ml^{-1} allowed initial growth of *A. hydrophila* but in lower densities as compared to those without probiotic. On the other hand, in higher concentrations of probiotic, *V. alginolyticus* S10 (10^6 CFU ml^{-1}), there was an increase in *A. hydrophila* densities during the first 2 days followed by a decrease in the total viable counts during the third and the fourth days then further increase in pathogen concentration was noticed in the fifth and sixth days but in lower levels corresponding to control without probiotics. Statistical analysis using t-test showed that, there were significant differences ($P< 0.05$) among the different treatments during the period of study. Therefore, it can be suggested that the inoculation of *V. alginolyticus* S10 strain into the rearing water in *M. japonicus* culture resulted in the apparent elimination of *Aeromonas* sp. Similarly, it was reported that *Pseudomonas fluorescens* reduced diseases caused by *Aeromonas solmonicidain*[48]. Also different probiotics were used to eliminate *Aeromonas hydrophila*[5].

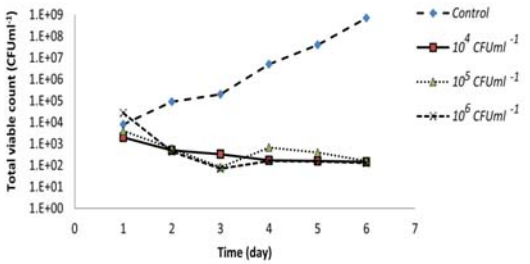

Fig. 4. Growth pattern of *A. hydrophila* without and with *V. alginolyticus* S10 at different concentrations (10^4 CFU ml^{-1}, 10^5 CFU ml^{-1} and 10^6 CFU ml^{-1})

Survival, behavior and histopathology

Temperature is often a major environmental factor in triggering waterborne disease outbreaks[49]. The ambient temperature in *M. japonicus* treatments and control group was 25 ± 1°C, the survival rate of *M. japonicus* attained 100% in treatment of adding the highest *V. alginolyticus* S10 concentration (10^6 CFU ml^{-1}). However, 88% survival rate was for both treatments of adding 10^4 CFU ml^{-1} and 10^5 CFU ml^{-1} *V. alginoticus* S10 and the lowest survival of 78% was recorded in control (without probiotics). This may be attributed to the fact that the ambient temperature (25±1°C) may be not enough for high mortality or severity pathogenesis as was previously reported[50].

In probiotic treatments, *M. japonicus* showed no abnormality in swimming behavior and appetite, while complete loss of appetite in control group was observed. The poor food consumption may lead to cannibalism[51]. The observed behavioral changes in *M. japonicus* may be attributed to the extracellular products of *A. hydrophila* as reported in *Penaeus indicus*[52].

Microscopic examination of shrimp tissues are among ideal and rapid reliable means for health monitoring and diseases diagnosis in farmed shrimp[53]. The present histopathological study that showed hepatopancreas (HP), muscle and heart lesser infection degrees in high concentrations of probiotic as compared to no/ low probiotic concentration indicates the positive probiotic impact against pathogenic bacteria revealing the probiotic use beneficiary.

The present study investigated the histopathology of (HP) since it is responsible for major metabolic events and plays a significant role in pathogenesis due to having open circulatory system[54, 55]. The present histological examination of fresh caught samples showed normal (HP) tissue containing numerous tubules that held together loosely by connective tissue strands to provide an increased surface area for digestion and absorption[54]. Each tubule has a star shaped lumen lined with various types of epithelial cells with clear nuclei, secretory vesicles and central lumen (Fig. 5-A).

It was reported that bacterial infections in shrimps may take various forms such as bacterial shell disease, localized gut or (HP) infections, localized infections from puncture wounds, limb loss *etc*. and septicemia[56]. The present histopathological investigations of infected *M. japonicus* (HP) showed necrotising hepatopancreas (NHP) disease. It is known that (NHP) targets penaeid shrimp, infecting the tubular epithelial cells of (HP)[57, 58] resulting in significant production losses, with 25 to 95% mortalities range[57, 59]. It also results in impairing the lipid storage, healthy growth, and development[60]. There are many reported signs for (NHP) such as bacterial shell disease (melanization)[61] which was detected by development of characteristic black coloration

in the exoskeleton of infected *M. japonicus*. It was noticed in control after exposure to *A. hydrophila* for three days however it was not observed in probiotic treatments. According to Lightner (1993)[62], the shell disease is characterized by single or multiple dark areas in general on body cuticle with brownish to black coloration. The black pigment deposit (melanin) is usually associates with the site of hemocytic activity and cellular inflammatory conditions during infection[63]. In addition, production of extracellular lipase, protease and chitinase enzymes may be among factors for the manifestation of shell disease [62, 64].

The (HP) tissue damage degree was noticed to be varied among treatments, it was found to be increased by increasing the exposure time to pathogenic *A. hydrophila* coinciding with decreasing probiotic concentration. Three stages of (NHP) have been defined in infected hepatopancreas. Stage I of (HP) infection was detected in control group (after one day), treatment 1 (10^4 CFU ml^{-1} *V. alginolyticus* addition) (after six days), and treatment 2 (after eight days). This stage was characterized by small, intra-cytoplasmic bacteria which result in B-cell vacuolation and interstitial hemocytic infiltration. Bacteria were limited to the tubular epithelium and were present in the apical cytoplasm (Figure. 5-B).

Stage II of (HP) infection was observed in control (after two days), treatment 1 (after seven days), and treatment 2 (at the end of experimental period, ten days). In stage II, severe vacuolation and rounding of B cells were noticed (Figure 5-C). Infected cells might appear cuboidal, contain little stored lipid vacuoles. B-cell vacuoles increased in number and some of them were extruded into the lumen. However, stage III of (HP) infection was detected only in control after seven days; it showed necrosis, degeneration of B-cells resulting in enlargement of tubule lumen, shrinkage and breakage of tubules (Figure 5-D). Likewise, it was stated that the acute phase of (NHP) by severely atrophied HP tubules[65]. The present findings are also in agreement with Ambipillai *et al* (2003)[31] who mentioned that heavy bacterial colonisation of the mouthparts or the cuticular lining of the

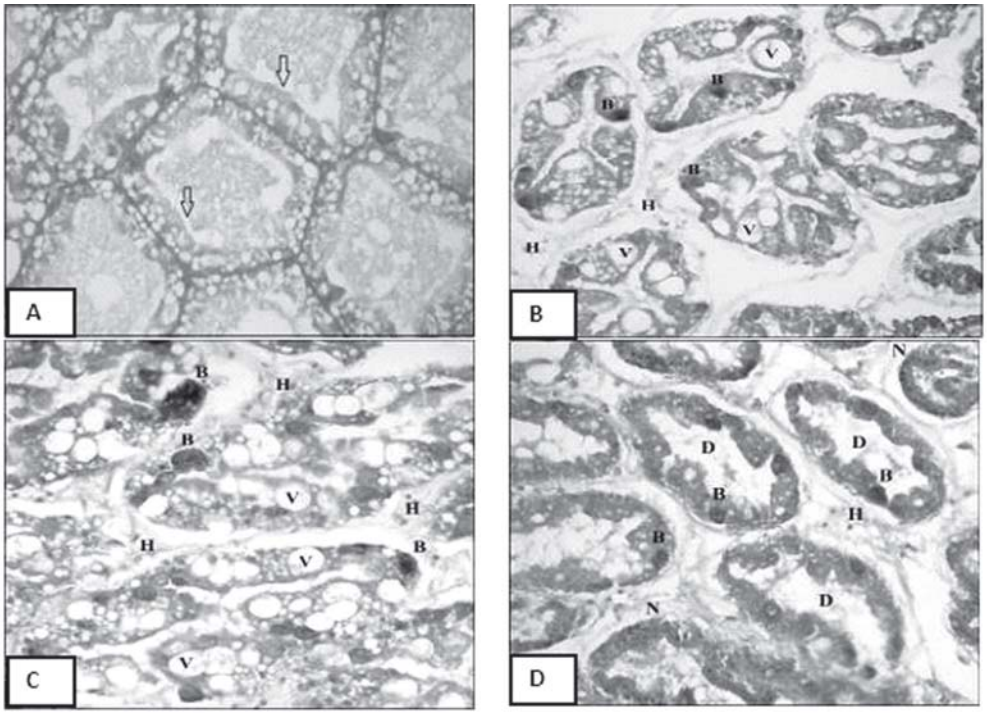

Fig. 5. Histological examination of *M. japonicus* hepatopancreas, A) normal hepatopancreas showing absence of intracellular bacteria in tubular epithelial cells (arrows). B) Stage I of hepatopancreas infection showing small, intra-cytoplasmic bacteria (B), interstitial hemocytic infiltration (H) and vacuolation (V), C) Stage II of hepatopancreas infection showing sever vaculation and rounding of cells, D) Stage III of hepatopancreas infection showing degeneration of B-cells (D), necrosis (N), and lumen enlargement (H & E: X, 400)

oesophagus and foregut can lead to rounding up and sloughing of (HP) tubules leading to typical enteritis.

The present histopathological observations of (HP), hemocytic infiltration, edema, and tubular atrophy with bacterial cytoplasmic masses within one or more tubules were similar to that recorded for (NHP) in *Litopenaeus vannamei*[66] and *P. monodon*[56]. The proposed route of pathogen invasion was stated previously[67] to be from stomach to the primary duct and the secondary duct, extending up to hepatopancreatic lumen.

Histological examination of fresh caught samples showed normal muscle tissue (Figure 6-A) while little tissue abnormality was observed in case of probiotic addition treatments (1, 2, and 3) (Figure 6-B). Damage of infected muscle tissue was represented by myofibril necrosis, interstitial hemocytic infiltration, and edema. The present finding of increased muscle tissue damage by increasing both of *A. hydrophila* densities and the exposure time is in agreement with that reported by Lightner (1973)[68] for *P. aztecus*.

Normal heart tissue without bacterial infection and hemolytic aggregation was noticed in fresh caught samples (Figure 7-A). Histopathological findings of infected *M. japonicus* heart tissue showed lesser hemocyte infiltration (Figure 7-B) in probiotic addition treatments (1, 2 and 3) in comparison to that sever damage observed in the control "without probiotic" after seven days of exposure (Figure 7-C). The destruction of heart tissue may be due to hepatopancreas autolysis and releasing of proteolytic enzymes was suggested in some studies[68].

The observed sever hemocyte aggregation in hepathopancreas, heart and muscles detected in control "without probiotics" may be attributed to stressful of pathogenic bacteria. The histopathological changes detected in infected heart and muscle tissues may speculate that bacteria may have reached them through open circulatory system as reported by Chavda & Sujata (2014)[1] for *Penaeus monodon* infected with *Fusarium sp.*

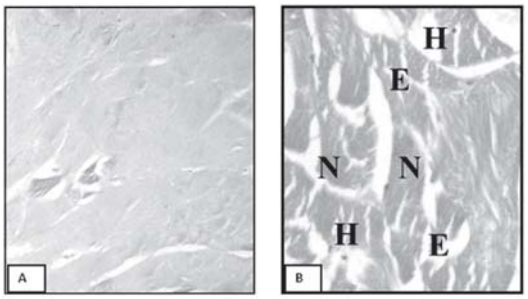

Fig. 6. Histological examination of *M. japonicus* muscle tissue, A) normal muscle tissue, B) infected *M. japonicus* muscle showing interstitial hemocytic infiltration (H), edema (E) and necrosis (N). (H&E: X, 400**)**

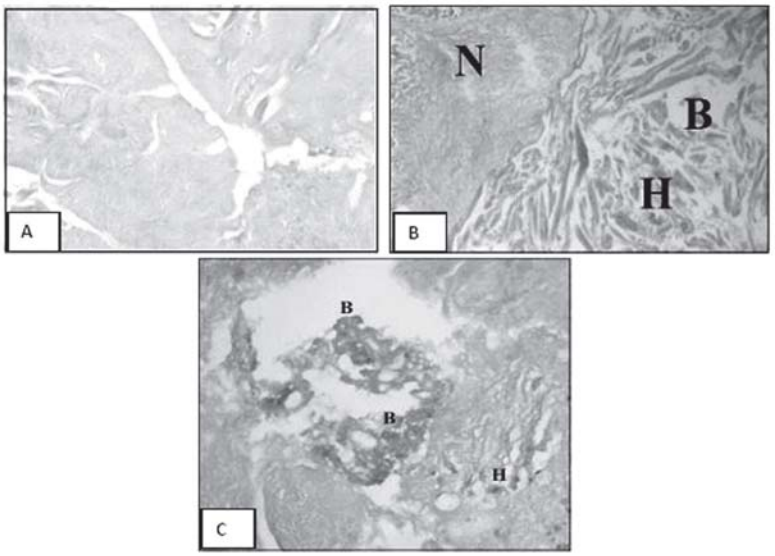

Fig. 7. Histological examination of *M. japonicus* heart tissue. A) normal heart tissue, B) Lesser infected heart tissue showing hemocyte aggregation, C) Sever infected heart tissue showing sever bacterial infection (B) with hemocyte aggregation (H) and necrosis (N). (H&E: X, 400)

REFERENCES

1. Chavda, D., Sujata, B. Occurrence of black gill disease in *Peneaus monodon* cultured in South Sujarat. Histopathology and antioxidant enzymes profile. *Life Sciences Leaflets.*,2014; **51** : 18-32.
2. Galil, B.S.: *Marsupenaeus japonicus* (Bate), Kuruma prawn (*Penaeidae, Crustacea*), in: DAISIE (Delivering Alien Invasive Species Inventories for Europe). Handbook of alien species in Europe, pp. 286. Invading Nature - *Springer Series in Invasion Ecology,* 2009; **3**.
3. FAO. "Species Fact Sheets: *Penaeus japonicus* (Bate, 1888)". FAO Fisheries and Aquaculture. Food and Agriculture Organization. 2012.
4. Downs, C., Fauth, J.E. and Woodley, C.M.. Assessing the health of grass shrimp (*Palaemonetes pugio*) exposed to natural and anthropogenic stressors: a molecular biomarker system. *Mar. Biotech.*2001*;* **3**: 380-397.
5. Pannu, R., Rani, R., Sarsar, V., Kumar, D. In vivo elimination of *Aeromonas hydrophila* from indian comman carp cyprinus carpio using probiotics. *Biomirror*, 2013**; 4**(10): 79-83.
6. Ramesh, K., Natarajan, M., Sridhar, H., Umamaheswari, S. Virulence determination among *Vibrio harveyi hatchery* isolates through haemolysis and growth constraint. *Glob. j. Bio-Sci. Biotechnol*., 2014**; 3**(1): 109-114.
7. Vaseeharan, B., Ramasamy, P. Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. *Lett. Appl. Microbiol*., 2003**; 36**: 83-87.
8. Lavilla-Pitogo, C.R. Bacterial disease of Penaeid shrimp. *Disease in Asian Aquacult*., 1995**;** II**:** 107-121.
9. Gonzalez, C.J., Encinas, J.P., Garcia-Lopez, M.L., Otero, A. Characterization of lactic acid bacteria from fresh water fishes. *Food.,* 2000**; 17:** 383.
10. Kent, M.L., Poppe, T.T. Infectious diseases of coldwater fish in marine and brackish water. In: Diseases and Disorders of Finfish in Cage Culture (P.T.K. Woo, D.W. Bruno, L.H.S. Lim, ed), CAB International Publisheds, USA, 2002; pp: 61-105.
11. Sung, H.H., Hsu, S.F., Chen, C.K., Ting, Y.Y., Chao, W.L. Relationship between disease outbreaks in cultured tiger shrimp (*Penaeus monodon*) and the composition of *Vibrio* communities in pond water and shrimp hepatopancreas during cultivation. *Aquaculture,* 2001**; 192**: 101-11.
12. Dahiya, T., Verma, R.K., Singh, G. Elimination of pathogenic bacterium, *Aeromonas hydrophila* by use of probiotics. *J. Fish.Sci*., 2012; **6**(3): 209-214
13. Xiong, Z., Wang, J., Hao, Y., Wang, Y. Recent advances in the discovery and development of marine microbial natural products. *Mar. Drug.*, 2013**; 11**(3):700–717.
14. Pham, D., Ansquer, D., Chevalier, A., Dauga, C., Peyramale, A., Wabete, N., Labreuche, Y. Selection and characterization of potential probiotic bacteria for *Litopenaeus stylirostris* shrimp hatcheries in New Caledonia. *Aquaculture*, 2014**; 432:** 475-482.
15. Thuy, H.T.T., Nga, L.P., Loan, T.T.C. Antibiotic contaminants in coastal wetlands from Vietnamese shrimp farming. *Environ. Sci. Pollut. Res. Int*., 2011**; 18**: 835–841.
16. Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W. Probiotic bacteria as biological control agents in aquaculture. *Microbiol. Molecul. Biolog. Res.*, 2000; **64**: 655-671.
17. Mishra, S., Mohanty, S., Pattnaik, P. Ayyappan, S. Probiotics - possible application in aquaculture. *Fish Chimes*, 2001; **21**: 31- 37.
18. Brunt, J., Austin, B. Use of a probiotic to control *lactococcosis* and *streptococcosis* in rainbow trout, Oncorhynchus mykiss(Walbaum). *J. Fish Diseas.*, 2005; **28**(12): 693-701.
19. Kamboj, V.P. Bioactive agents from the ocean biota, in: Ocean science trends and future directions (B.L.K. Somayajulu, ed), Indian National Science Acadamy, New Delhi, 1999; pp 197-227.
20. Rinaudo, M. Chitin and chitosan: Properties and applications. *Progr. Polym. Sci.*, 2006; **31**(7): 603–632
21. No, H.K., Park, N.Y., Lee, S.H., Meyers, S.P. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights," *Int. J. Food Microbiol*., 2002**; 74:** 65-72.
22. Abo-Elela, G.M., El-Sersy, N.A., El-Shenawy, M., Abdel-Mawla, E.A. Microbial assessment of El Max Fish Farm. *Egy. J. Aqua. Res*., 2005; **31**: 171-184.
23. Rattanachuay P., Kantachote D., Tantirungkij M., Nitoda T. and Kanzaki H. Inhibition of shrimp pathogenic vibrios by extracellular compounds from a proteolytic bacterium *Pseudomonas* sp. W3. *Electronic J. Biotechnol*., 2010 **; 13**(1), ISSN: 0717-3458.
24. Ramasamy M., Sukumaran, V., yyavooV, Allam, T. Potential antibacterial activity of marine bivalves *Meretrix casta* and *Tridacna maxima* from south east coast of India. *Adv. Biores*..,

2010**; 1**: 92-96.

25. Islam, M.M., Masumb, S.M., Rahmana, M.M., Islam Mollab, M. A., Shaikhc, A.A., Roya, S.K. Preparation of chitosan from shrimp shell and investigation of its properties. *Int. J. Basic Appl. Scien.*, 2011**; 11**(01): 116-130.
26. El-Masry, M.H., Khalil, A.I., Hassouna, M.S., Ibrahim, H.A. In situ and in vitro suppressive effect of agricultural composts and their water extracts on some phytopathogenic fungi. World. *J. Microbiol. Biotechnol*., 2002; **18**: 551-558.
27. Abou-Elela, G.M., El-Sersy, N.A., El-Shenawy, M.A., Abd-Elnaby, H.M. Ibrahim, H.A.H. Bio-Control of *Vibrio fluvialis* in aquaculture by mangrove (*Avicennia marina*) seeds extracts. *Res. J. Microbiol.*, 2009; **4**(1): 38-48.
28. Hall, T.A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT. *Nucl. Acids Symp. Ser.*, 1999; **41**: 95-98
29. Lightner, D.V. A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. The World Aquaculture Society, Baton Rouge, Louisiana. 1996.
30. Zar J.H. Biostatistical analysis. Englewood Cliffs, NJ: Prentice-Hall. 1984; pp. 718.
31. Ambipillai, L., Sobhana, K.S., George, K.C., Sanil, N.K. Histopathological survey of cultured shrimps in Cochin, Kerala. *J. Mar. Biolog. Associat. India*, 2003; **45**(2): 178 -185.
32. Pereira, M.A., Nunes, M.M., Nuernberg, L., Schulz, D., Batista, R.V. Microbiological quality of oysters (*Crassostrea gigas*) produced and commercialized in the coastal region of Florinopolis Brazil. *Braz. J. Microbiol.*, 2006**; 37**:159-163.
33. Armstrong, E., Yan, L., Boyd, K.G., Wright, P.C., Burgess, J.G. The symbiotic role of marine microbes on living surfaces. *Hydrobiologia*, 2001; **461**: 37-40.
34. Fernandes, J.C., Tavaria, F.K., Soares, J.C., Ramos., Igbinosa, E.O., Okoh, A.I. Emerging *Vibrio* species: an unending threat to public health in developing countries. *Res. Microbiol*., 2008 **; 159:** 495-506.
35. Adhikari, H., Ali, M.Y., Shahiduzzaman, M., Ibn Shams, F., Sarower, M.G. Biochemical and PCR assay for detection of pathogenic bacteria at shrimp and shrimp farms in Bangladesh. *Fish Aquac. J.*, 2015; **6**(2):1–10.(3): 273-276.
36. Popovic, T.N., Benussi Skukan, A., Dzidara, P., CozRakovac, R., Strunjak-Perovic, I., Kozacinski, L., Jadan., M., Brlek-Gorski, D. Microbiological quality of marketed fresh and frozen seafood caught off the Adriatic coast of Croatia. *Vet Med-Czech*, 2010; **55**: 233-241
37. Oxley, A.P.A., Shipton, W., Owens, L., McKay, M.D. Bacterial flora from the gut of the wild and cultured banana prawn, *Penaeus merguiensis. J. Appl. Microbiol.*, 2002**; 93**: 214–223.
38. Ripabelli, G., Sammarco, M.L., Grasso, G.M., Fanelli, I., Caprioli, A., Luzzi, I. Occurrence of *Vibrio* and other pathogenic bacteria in shrimp (*Penaeus monodon*) and the composition of *Vibrio* communities in pond water and shrimp hepatopancreas during cultivation .*Aquaculture*, 1999; **192:** 101–11.
39. Burgess, J.G., Elizabeth, M., Jordan, Breg Andrew, M., Mearns-Spragg, Kenneth,M., Boyd. G. Microbial antagonism: a neglected avenue of natural products research. *J, Biotechnol*., 1999 ; **70**: 27–32.
40. Zheng, L., An, R., Wang, J., Sun, N., Zhang, S., Hu, J., Kuai, J. Exploring novel bioactive compounds from marine microbes. *Curr. Opin. Microbiol*., 2005; **8**: 276-281.
41. Neill, j.S., Desikan, R., Clarke, A., Hancock, J.T. Novel component of abscisic acid signaling in stomatal guard cells. *Plant Physiolog.,* 2002**; 128**(1):13-16.
42. Argente, F.A.T., Ilano A.S. Antibacterial activity of the Mud Clam, *Polymesoda expansa* (Mousson 1849) (Bivalvia: Corbiculidae). *Banwa A*, 2014; **11**.
43. Sukumaran, R.M.V., Ayyavoo, M. Potential antibacterial activity of marine bivalves *Meretrix casta* and *Tridacna maxima* from south east coast of India. *Adv. Biores*., 2010**; 1**(1): 92–96.
44. Abu Tareq, A,M., Raza, S., Sarwar, T., Fardous, Z., Alamgir, Z., Chowdhury, Hossain, S. Comparative study of antibacterial activity of chitin and chemically treated chitosan prepared from shrimp (*Macrobrachium Rosenbergii*) shell waste. *J. Virol. Microbiol*., 2013; 1-9 , Article ID 369217
45. Peter, X.M. Biomimetic materials for tissue engineering. Advan. *Drug Delivery Rev.*, 2008**; 60**: 184–198.
46. Farzanfar, A. The use of probiotics in shrimp aquaculture. *FEMS Immunol Med. Microbiol*., 2006; **48**(2): 149-58.
47. Kersarcodi-Watson, A., Kaspar, H., Lategan, M.J., Gibson, L. Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. *Aquaculture*, 2008; **274**:1-14.,
48. Smith, P., Davey, S. Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress-inducible furunsculosis by a *fluorescent Pseudomonad. J. Fish Diseas*., 1993; **1**: 521–524.

49. Tang, Y., Tao, P., Tan, J., Mu, H., Peng, L., Yang, D., Tong, S., Chen, L. Identification of bacterial community composition in freshwater aquaculture system farming of *Litopenaeus vannamei* reveals distinct temperature-driven patterns. *Int. J. Mol. Sci.*, 2014**; 15**:13663-13680.
50. SamCookiyaei A., Afsharnasab M., Razavilar V., Motalebi A. A.,Kakoolaki S., Asadpor Y., Yahyazade M. Y.,Nekuie Fard A. Experimentally pathogenesis of *Aeromonas hydrophila* in freshwater Crayfish (*Astacus leptodactylus*) in Iran . *IRAN-J-FISH-SCI*.2012; **3**: 644 - 656.
51. Soyl, H. L., Kumlu, M. The effects of salinity on postlarval growth and survival of *Penaeus semisulcatus* (Decapoda: Penaeidae). *Turk. J. Zool*., 2003; **27**: 221–225.
52. Gopinath, R., George, K.C. The haemolymph response of *Penaeus indicus* to the extracellular products of *Aeromonas hydrophila. J. Mar. Biol. Ass. India*, 2000; **42**(1-2): 84-90.
53. Sparks, A. K. Synopsis of invertebrate pathology exclusive of insects.1985. Elsevier Science Publishers, Amsterdam.
54. Gibson, R., Barker, P.L. The decapod hepatopancreas. Oceanogr. *Mar. Biol..*, 1979; **17**:285-346.
55. Rameshthangam, P., Ramasamy, P. Antioxidant and membrane bound enzymes activity in WSSV-infected *Penaeus monodon* Fabricius; *Aquacul.*, 2006; **254**:32-39.
56. Abraham, T.J., Sasmal, D., Dash, G., Nagesh, T.S., Das, S.K., Mukhoopadhayay, S.K., Ganguly, S. Epizootology and pathology of bacterial infections in cultured shrimp *Penaeus monodon* Fabricius 1798 in West Bengal, India. *Indian J. Fish.*,2013; 167-171.
57. Loy, J. K., Dewhirst, F. E., Weber, W., Frelier, P. F., Garbar, T. L., Tasca, S. I., Templeton, J. W. Molecular phylogeny and *in situ* detection of the etiologic agent of necrotizing hepatopancreatitis in shrimp. *Appl. Environ. Microbiol.*, 1996; **62** : 3439-3445.
58. Bradley-Dunlop D. J., Pantoja C.R., Lightner, D.V. Development of monoclonal antibodies for the detection of necrotizing hepatopancreatitis in penaeid shrimp. *Dis. Aqu, Org..*, 2004; **60**: 233–240.
59. Gomes, G. B., E. S. Mendes, and V. A. Silva**.** Hepatopancreatite Necrosante (NHP) em camar˜ao marinho: revis˜ao de literatura. *Ci ̂encia Veterin´aria nos Tr´opicos*., 2007; **10**: 17–19.
60. Aranguren, L. F., B. Bri ˜ nez, L. Arag´on, C. Platz, X. Caraballo, A. Suarez, and M. Salazar**.** Necrotizing hepatopancreatitis (NHP) infected *Penaeus vannamei* female broodstock: effect on reproductive parameters, nauplii and larvae quality. *Aquacul*., 2007; **258**: 337–343.
61. Aguirre-Guzmán, G, Ascencio Valle, F. Infectious disease in shrimp species with aquaculture potential. In: Pandalai SG (ed) Recent research developments in microbiology, 2000; Research Signpost, Kerala, p 333–348
62. Lightner, D.V. Disease of cultured penaeid shrimp. In: CRC handbook of mariculture, 2nd ed., Vol.1, Crustacean Aquaculture (J.P. McVey, ed), CRC Press Inc. Boca Rough, FL. 1993; pp. 393-486.
63. Lightner, D.V., Redman, R. Histochemical demonstration of melanin in cellular inflammatory processes of penaeid shrimp. *J. Inv. Patho.*,1977; **30**: 298-302.
64. Abraham, T.J., Manley, R. Luminous and non-luminous *Vibrio harveyi* associated with shell disease in cultured *Penaeus indicus. J. Aquacult. Trop*., 1995; **10.**
65. Morales-Covarrubias, M. S. Enfermidades del camar´on: detecti´on mediante an´alisis en fresco ehistopatología. Trillas, 2004; Mexico City, Mexico.
66. Gomes, G.B., Domingos, S., Oliveira, K.C., De Paula Mendes, P., Da Silva, V.A., Mendes, E.S. Diagnosis of Necrotizing Hepatopancreatitis in Pacific White Shrimp, *Litopenaeus vannamei*, through Wet Mount, Histopathology and PCR Techniques. WAS., 2010; **5**: 816-822.
67. Chen, S.N., Huang, S.L., Kou, G.H.: Studies on the epizootology and pathogenicity of bacterial infections in cultured giant tiger prawns, *Penaeus monodon* in Taiwan, in: Diseases in cultured *penaeid* shrimp in Asia and the United States (W. Fulks and K.L. Main ,ed,), . The Oceanic Institute, Honolulu, Hawaii, 1992; pp. 195-205
68. Lightner, D.V. Normal postmortem changes in the brown shrimp, *Penaeus aztecus*. Fish. Bull., 1973. **72**: 223-236.