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Effect of WSSV-Infection on Relative Expression of Hemocyanin Genes in *Litopenaeus Vannamei* (Boone, 1931)

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***Abstract* - A *Litopenaeus vannamei* injected with the serum from the shrimps infected with WSSV-showed the typical symptoms of white spot syndrome at 48hpi. These include lethargy, lack of appetite, reduced and disoriented swimming activity. The negative control and serum from healthy shrimps did not show any band and the test samples contained a band corresponding to 310 bp which was like that of the positive control. Thus, the test samples were confirmed positive for the WSSV-infection. The mean serum protein concentration in healthy shrimp was 86.4 ± 6.3 mg/ml. on the other hand the serum protein concentration of WSSV-injected shrimps declined linearly and significantly to 58.8 ± 3.9, 46.2 ± 5.32, and 38.7 ± 6.7 mg/ml at 12,24, and 48 hpi, respectively. Relative quantity of hemocyanin gene (I) expression in WSSV-injected shrimps increased gradually from 6 to 48 hpi. Analyses of relative quantification of hemocyanin gene (II) in the experimental groups showed that the relative quantity in WSSV- injected shrimps increased from 6 hpi up to 24 hpi, whereas at 48 hpi the WSSV-injected shrimps showed a decrease in relative quantity of hemocyanin gene (II). Analyses of relative quantification of hemocyanin gene (III) in the experimental groups showed that the relative quantity in WSSV-injected shrimps increased slowly from 6 to 12 hpi and the increase was found to be high at 24 and 48 hpi. The relative quantity increased in sham-injected shrimps. The melt curve analysis showed two peaks corresponding to 18S rRNA and hemocyanin gene I, II and III, respectively. The objective of the present study was directed towards the relative quantification of the hemocyanin genes (I, II and III) in *L. vannamei* upon WSSV-infection.**
Keywords: **Hemocyanin Gene, *Litopenaeus vannamei,* WSSV**

I. INTRODUCTION

The respiratory protein, hemocyanin is the most abundant protein, constituting about 60-95% of the total proteins in crustacean haemolymph (Djangmah, 1970; Balaji *et al.,* 1989; Noga, 2000) followed by the clottable protein (Lynch & Webb, 1973). The internal tissues and cells are bathed in haemolymph, therefore, any change or alternation in the internal environment due to adverse environmental, physiological, or pathological conditions would reflect in the profiles of various haemolymph components. The molecules released by tissues or cells under such conditions can be detected in the haemolymph. Among these haemolymph components, total proteins serve as a reliable health marker for crustaceans. The total protein concentrations are known to be influenced in crustaceans by variations in sex (Horn & Kerr, 1963; Adiyodi, 1968), nutritional state (Djangmah, 1970), moult stages (Leone, 1953), and most importantly upon pathogenic infections. Earlier findings reveal a wide variation in the protein concentration within and among closely related groups of crustaceans.

Besides, the impact of infections including increase, decrease, or no change in haemolymph protein concentration is evident from these studies. Previously many investigators have reported on the effect of various pathogens on the total protein concentration in different haemolymph fractions of crustaceans. Such pathogens include bacteria, virus, fungi, protozoans and other parasites. Among the penaeid shrimps, *P.monodon*, *V.vulnificus* infection increased the serum protein concentration by 64% (Ratanapo & Chulvatanol, 1992).

On the other hand, Ramalingam & Ramarani (2006) reported that there was no change in haemolymph protein during *P. aeruginosa* infection. Contrastingly a decrease in plasma protein was reported in *P. semisulcatus* during infection (Mohajeri *et al.,* 2011) and Hose *et al.,* (1984) have reported a decrease in serum protein by 34% during *F. solani* infection in *F. californiensis*. Among various pathogens listed above, viruses are most devastating pathogens to penaeid shrimps. Viruses like Taura Syndrome Virus, Infectious Myo Necrosis Virus, and White Spot Syndrome Virus are common viruses which affect the shrimps. These virus infections are known to deplete the total protein concentrations during the infections (Song *et al.,* 2003; Jiang, 2010).

Despite many studies were focused on quantitative analysis of haemolymph proteins in WSSV-infected shrimps, qualitative analysis is limited (Rameshthangam & Ramasamy, 2005; Pais *et al.,* 2007). The transport of oxygen and carbon dioxide is essential for efficient aerobic respiration in most metazoans. A variety of respiratory pigments have evolved to facilitate this process, and they are invariably metal-containing proteins namely, haemoglobins, hemerythrins, and hemocyanins (Bonaventura & Wood, 1980). The site of oxygen binding is entirely different in each of the three classes of respiratory proteins: a single Fe (II)

contained in a heme group in haemoglobins, two Fe (II) atoms bound by amino acid side chains in hemerythrins, and two Cu (I) atoms bound directly to side chains in hemocyanins. Of these respiratory pigments, the hemocyanins are widely present in numerous species of arthropods and molluscs (Mangum, 1997; Hodgson & Spicer 2001).

Among the arthropods, four classes, namely, onychophorans, myriapods, chelicerates, and crustaceans, possess hemocyanin. Interestingly, the presence of hemocyanin in an insect has been demonstrated in the haemolymph of nymphs and adults of the stone fly, *Perla marginata* (Hagner-Holler *et al.,* 2004). In molluscs, this respiratory protein appears to occur in three classes including Amphineura, Gastropoda, and Cephalopoda, but not in Lamellibranchiata and Scaphopoda. Thus, hemocyanin is distributed in many disparate groups of invertebrates and it is also considered as a model protein to resolve structure-function relationships as well as assembly properties of large multi subunit proteins. A 45-day feeding trial followed by an acute stress test of low salinity was done to evaluate effects of Lactobacillus *plantarum* on growth performance and anti-stress capability of white shrimp, *Litopenaeus vannamei* (Zheng *et al.,* 2017).

Micro RNAs (miRNAs) are small non-coding RNAs that regulate diverse cellular processes, including organismal stress response, through posttranscriptional repression of gene transcripts. They are known to have antiviral functions in aquatic crustacean species, but little is known about the role of miRNAs against environmental stress caused by Cu, a common chemical contaminant in aquatic environment. We performed small RNA sequencing to characterize the differentially expressed microRNAs in Cu exposed shrimp (Guo *et al.,* 2018).

II. MATERIALS AND METHODS

A. Experimental Animal: Collection and Maintenance

The white leg shrimp, *Litopenaeus vannamei* with an average body weight of about 13 ± 2 g were collected from shrimp farms, in and around Chennai. These shrimps were transported in plastic buckets with battery powered aerators and were acclimatized in sea water (approximately 35‰) in the laboratory with continuous aeration until use. The water was changed every day and the shrimps were fed ad libitum with pelletized feed. The animals were acclimatized for one week in the laboratory and only intermoult animals irrespective of size and sex were used for further studies.

B. Experimental Design

In the present study, the shrimps were divided into three groups namely healthy, sham-injected and WSSV- infected serum injected. The shrimps were acclimatized under the laboratory conditions for a week and the expression of hemocyanin genes (I, II and III) were analysed at 6, 12, 24 and 48 hours of post injection.

C. Experimental Infection of Shrimps with WSSV

The shrimps were experimentally infected with WSSV by intramuscular injection of 100µl of serum from the WSSV – infected shrimp on the lateral side between the third and fourth abdominal segments using sterile 1-ml insulin syringe. The initial symptoms of WSSV were detectable at 24 hrs of post-injection (hpi) and they reached the terminal (moribund) stage within 48 hpi. The shrimps injected with the same volume of WSSV-negative serum obtained from healthy shrimps served as sham-injected animals. Haemolymph samples and hepatopancreas from healthy, sham-injected and WSSV-inoculated shrimps were collected at 6, 12, 24, 48 hpi for determination of protein concentration in this study. The haemolymph samples collected from WSSV-inoculated shrimps at different time intervals were also subjected to analysis for the presence of WSSV and quantification of hemocyanin gene (I, II and III) expression using real time quantitative PCR.

D. Separation of Serum

Haemolymph samples from the *Litopenaeus vannamei* during the experimental period were collected by cardiac puncture using a sterile syringe. The haemolymph samples were collected and transferred to micro centrifuge tubes held on ice and allowed to clot for 30 minutes at room temperature (28 ± 2˚C). The clot was disturbed using a clean glass rod and then centrifuged at 1500 rpm for 7 minutes. The clear supernatant (= serum) was collected and used for further studies.

E. Serum Osmolality

The serum osmolality of five intermoult shrimps from different groups of shrimps was measured using cryoscopicosmometer (osmomet 030- Gonotec). 50 µl of serum, which was retrieved from the haemolymph as mentioned above were used for osmolality analysis.

F. Preparation of WSSV Inoculum

WSSV-infected *Penaeus monodon* with prominent white spots on the cephalothoracic region due to natural WSSV infection were collected from Pulicat Lake, near Chennai. The haemolymph from each shrimp was drawn directly from the heart using a sterile syringe and centrifuged (1500 rpm, 10 min, 4^{O}C). After testing for WSSV-positivity by nested PCR, the supernatant was aliquoted, stored at -20C and used as WSSV inoculum for subsequent infectivity studies within two weeks.

G. Quantitative Estimation of Protein

Protein concentration in the whole serum was estimated by the method of Lowry *et al.,* (1951) using bovine serum albumin (BSA) dissolved in 1 N sodium hydroxide as standard. The protein concentration of the serum from healthy, sham- and WSSV-injected *L. vannamei* was

determined in whole serum with alcohol-precipitation. Briefly, 25 µl of the serum sample was added to 2 ml of 80% ethanol and mixed well, and the white precipitate formed was centrifuged (400 x g for 5 min).

The supernatant was discarded and 1 ml of 1 N sodium hydroxide was added to dissolve the precipitate. After 10 minutes, 0.5 ml of Folin phenol reagent was added and mixed well. In blank and standard, the serum samples were substituted by 1 ml of 1 N sodium hydroxide and 25 µl of standard protein solution (BSA) respectively. All the samples were incubated for 30 minutes at room temperature. The absorbance of the sample and the standard were measured at 500 nm using Shimadzu (UV-160A) spectrophotometer against the reagent blank.

H. DNA Extraction

DNA isolation was achieved using the DNA extraction buffer supplied in the Dr. Shahul's nested Polymerase Chain Reaction kit. 100 µl of serum sample from individual shrimp was taken in 600 µl of DNA extraction buffer and allowed to mix in the same buffer for 10 minutes at room temperature. The sample was then centrifuged at 5000 rpm for 5 min and 200 µl of the supernatant was transferred to another tube.

The DNA was precipitated by addition of 2 volumes of 100% ethanol (EtOH) and sample mixed well. Samples were centrifuged at 10,000 rpm for 10 min to pelletize the DNA. The supernatant was discarded leaving the DNA pellet at the bottom of the tube. Pelletized DNA was dissolved in 100µl of sterile H_2O by mixing gently with a micro pipette. This served as template for further reactions in PCR.

I. First PCR

A PCR tube containing PCR master mix was taken and to this 6.5µl of first primer mix and 1.0µl DNA template was added to PCR tube. The reaction mixture was mixed gently and placed in the PCR machine. The PCR was programmed for pre-denaturation temperature at 95ºC for 5 minutes and 30 cycles of denaturation temperature at 95ºC for 30 seconds and annealing temperature at 57ºC for 30 seconds. Extension temperature at 72ºC for 1 minute, post extension temperature was maintained at 72ºC for 10 minutes and hold at 4ºC was maintained.

J. Nested PCR

A PCR tube containing PCR master mix was taken and to this 6.5µl of nested primer mix and 1.0µl of DNA template was added, mixed gently and placed into the PCR machine. The PCR is programmed for pre- denaturation temperature at 95ºC for 5 minutes and 30 cycles of denaturation temperature at 95ºC for 30 seconds, annealing temperature at 60ºC for 30 seconds, extension temperature at 72ºC for 30 seconds, post extension temperature at 72ºC for 10 minutes and hold at 4ºC was maintained.

K. Isolation of Total RNA from the Hepatopancreas of Healthy, Sham- And WSSV-Injected L. Vannamei

The total RNA from the hepatopancreas of *L. vannamei*was isolated using RNAisoplus reagent. In this method the hepatopancreas (whole) was dissected out from the animal and homogenized completely. To this homogenate 1 ml of RNA isoplus reagent was added and incubated for 5 min at room temperature. This mixture was centrifuged at 10,000 rpm for 5 min at 4ºC. The resultant supernatant was transferred to a new centrifuge tube, then 0.8 ml of chloroform was added to the supernatant obtained in the previous step and the mixture was vortexed vigorously and kept at room temperature for 5 min. This mixture was centrifuged at 10,000 rpm for 15 min at 4ºC. After centrifugation the upper layer was transferred to a new centrifuge tube and 4 ml of isopropanol was added and kept at room temperature for 10 min. The reaction mixture was centrifuged at 10,000 rpm for 10 min at 4ºC. Equal volume of 75% ethanol was added to this reaction mixture and centrifuged at 8,000 rpm for 5 min at 4ºC. Supernatant was discarded, and the precipitate was air dried. Precipitated RNA was dissolved in 100 µl of sterile water.

L. Synthesis of cDNA

The synthesis of first strand cDNA from the total RNA isolated from the hepatopancreas of healthy, sham- and WSSV-injected *L. vannamei* has been carried out using Revert Aid first strand cDNA synthesis kit (Thermo Scientific). After thawing, the components of the kit were centrifuged briefly and stored on ice. Template RNA (0.1ng – 5 µg), primer (random hexamer: 1µl) were added in PCR tube and the reaction volume was made up to 12 µl with sterile water. To this reaction mixture, 4µl of reaction buffer, 1 µl of Ribo Lock RNase inhibitor, 2 µl of 10mM dNTP mix and 1µl of Revert Aid M- MuLV RT were mixed in order and centrifuged briefly. The reverse transcriptase reaction has been carried out in the following order.

M. Quantification of Expression of Hemocyanin Genes I, II and III from the Hepatopancreas of L. Vannamei

The quantification of hemocyanin genes (I, II and III) from the hepatopancreas of the healthy, sham- and WSSV-injected *L. vannamei* has been carried out using real time quantitative PCR. Briefly, 5 µl of master mix, 1 µl forward primer, 2 µl of reverse primer, 1 µl of cDNA (50ng) and 2 µl of nuclease free water were added in order and to this total reaction mixture 0.2 µl of SYBR fast reagent were added. 18 S rRNA gene were used as endogenous control in the present reaction.

N. Agarose Gel Analysis

Gel was prepared by melting agarose in 1 X TAE buffer in a microwave oven, mixed and cooled to 50-55ºC. Ethidium bromide (EtBr) was added to agarose gel solution before pouring into the gel casting platform. The gel was casted by carefully pouring agarose solution into the sealed gel casting platform, and gel comb was inserted. The gel was covered only about 1/3 of the height of the comb teeth, using a pipette

tip to move larger bubbles or solid debris to the sides or the end of the tray, while the gel was in liquid state. After the gel was hardened, the seal from the gel casting platform was removed and the gel comb was withdrawn. The casted gel was placed into an electrophoresis tank and filled with 1 X TAE buffer. After the completion of PCR, the tubes were centrifuged briefly in micro centrifuge and the samples were loaded in wells in gel, 3µl of molecular weight marker was loaded in outer lane. The lead was attached for the DNA migration from anode to positive lead. Power supply was turned- off when the loading dye has migrated a distance judged sufficient for separation of the DNA fragments. Then the gel was viewed under the UV transluminator and the results were interpreted.

O. Statistical Analyses

The difference between healthy, sham- and WSSV-injected samples were analysed for statistical significance using Student t – test (Bailey, 1994). Mean difference t – test was employed depending on the sampling procedure followed for each set of experiment.

III. RESULTS OF THE STUDY

A. Infection of Shrimps with WSSV

Litopenaeus vannamei injected with the serum from the shrimps infected with WSSV- showed the typical symptoms of white spot syndrome at 48 hpi. These included lethargy, lack of appetite, reduced and disoriented swimming activity. Subsequently other parts of the body like their uropods, telson, periopods and pleopods became reddish in colour. White spots were prominently seen on the cephalothoracic region of almost all these animals at moribund stage with 98 to 100% mortality. Nested PCR analysis invariably showed positive for WSSV in the serum samples collected from these moribund animals whereas both healthy and sham-injected shrimps did not show any of the above symptoms and survived well during the experimental period. And their test samples were negative for WSSV.

B. Detection of WSSV using Nested PCR

The shrimps were injected with 100µl of the WSSV- viral inoculum prepared as mentioned in materials and methods. The presence of virus in the serum was confirmed by Dr. Shahul's nested Polymerase Chain Reaction kit. The PCR product was analyzed by running 1% agarose gel electrophoresis. As shown in Figure 1 the negative control and serum from healthy shrimps did not show any band and the test samples contained a band corresponding to 310 bp which was like that of the positive control. Thus, the test samples were confirmed positive for the WSSV-infection.

C. Analysis of Serum Protein Concentration in Shrimps upon WSSV-Infection

The total serum protein concentration was determined in healthy, sham- and WSSV-injected *L. vannamei* at various time points of post-injection. As shown (Fig. 2), the mean serum protein concentration in healthy shrimps was 86.4 ± 6.3 mg/ml. On the other hand, the serum protein concentration of WSSV-injected shrimps declined linearly and significantly to 58.8 ± 3.9, 46.2 ± 5.32, and 38.7 ± 6.7 mg/ml at 12, 24 and 48 hpi, respectively. The decline was statistically significant from 12 hpi onwards ($p < 0.05$) compared to healthy shrimps. The serum protein content decreased and reached 44% of the protein content in the serum from healthy *L. vannamei* at the terminal stage (48 hpi) of WSSV-infection.

D. Isolation of Total RNA from the Hepatopancreas of Healthy, Sham- and WSSV-Injected L. Vannamei

The total RNA from the hepatopancreas of shrimps has been isolated using RNA isoplus reagent. The RNA isolated was analysed on 1% agarose gel for the confirmation. The results confirmed the presence of adequate quantity of RNA.

E. Synthesis and Quantification of cDNA

The first strand of cDNA was synthesised from the RNA isolated in the previous step by using Revert Aid First strand cDNA synthesis kit. The cDNA synthesised was quantified and run on 1% agarose gel for the confirmation.

F. Analysis of mRNA Expression and Relative Quantification of Hemocyanin I Gene in the Hepatopancreas from Healthy, Sham- and WSSV-Injected L. Vannamei at 6, 12, 24 and 48 hpi.

The results pertaining to the threshold cycle values for the analysis of mRNA expression of hemocyanin gene I was represented in The mean of threshold cycles for the quantification of 18S rRNA ranged from 18 to 34. On the other hand, the mean of threshold cycle for cDNA from healthy, sham- and WSSV-injected *L. vannamei* ranged from 12.8 to 39.5. Relative quantity of hemocyanin gene I expression in WSSV-injected shrimps increased statistically significant when compared to healthy shrimps. In the present experiment hemocyanin gene I expression in sham-injected shrimps was high when compared to healthy shrimps. PCR products were analysed on the 1% agarose gel which showed a single band corresponding to hemocyanin gene I (Fig. 3). The melt curve analysis showed two peaks corresponding to 18S rRNA and hemocyanin gene I (Fig. 4).

G. Analysis of mRNA Expression and Relative Quantification of Hemocyanin Gene II in the Hepatopancreas from Healthy, Sham- and WSSV-Injected L. vannamei at 6, 12, 24 and 48 hpi.

The mean of threshold cycles for the quantification of 18S rRNA and hemocyanin gene II in healthy, sham- and WSSV-injected *L. vannamei* are represented in Mean Ct value for the 18S rRNA in healthy shrimps was 19.08 and maximum threshold cycle was detected for sham-injected shrimps at 48 hpi. On the other hand, the mean of threshold cycle for

hemocyanin II gene healthy, sham- and WSSV-injected shrimps ranged from 21 to 35. Analyses of relative quantification of hemocyanin gene II in the experimental groups showed that the relative quantity in WSSV-injected shrimps increased from 6 hpi up to 24 hpi. Whereas at 48 hpi the WSSV-injected shrimps showed a decrease in relative quantity of hemocyanin gene II. On the other side increase in relative quantity of hemocyanin gene II was also noticed in sham-injected shrimps. The PCR products were analysed on 1% agarose gel. The analyses showed the presence of single corresponding to the target gene (Fig. 5). The melt curve analysis showed two peaks corresponding to 18S rRNA and hemocyanin gene II (Fig. 6).

H. Analysis of mRNA Expression and Relative quantification of Hemocyanin Gene III in the Hepatopancreas from Healthy, Sham- and WSSV-Injected L. vannamei at 6, 12, 24 and 48 hpi.

The mRNA expression and relative quantification of hemocyanin III gene was carried in the shrimps at 6, 12, 24 and 48 hpi. In the mean of threshold cycles for the quantification of 18S rRNA and hemocyanin gene III in healthy, sham- and WSSV-injected *L. vannamei* are represented. Healthy shrimps showed a mean Ct value of 23.5 for the 18S rRNA. Mean threshold cycles for sham-injected shrimps were 32.9, 32.3, 32.6, and 34.5 at 6, 12, 24 and 48 hpi. On the other hand, the mean of threshold cycle for hemocyanin III gene in healthy, sham- and WSSV-injected shrimps ranged from 18 to 36.2.

Analyses of relative quantification of hemocyanin gene III in the experimental groups showed that the relative quantity in WSSV-injected shrimps increased slowly from 6 to 12 hpi and the increase was found to be high at 24 and 48 hpi. On the other hand, the relative quantity increased in sham-injected shrimps. The PCR products were analysed on 1% agarose gel. The analyses showed the presence of single band corresponding to the target gene (Fig. 7). The melt curve analysis showed two peaks corresponding to 18S rRNA and hemocyanin gene III (Fig. 8).

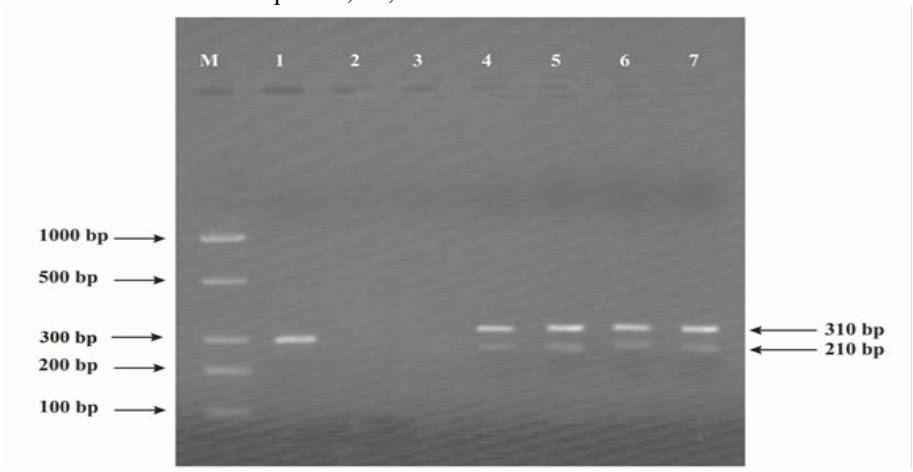

Fig. 1 Detection of WSSV in the serum of *Litopenaeusvannamei*.

M: DNA Marker, 1: Positive control, 2: Negative control, 3: Serum from healthy Shrimp, 4: Serum from WSSV-injected shrimp at 6 hpi, 5: Serum from WSSV-injected shrimp at 12 hpi, 6: Serum from WSSV-injected shrimp at 24 hpi, 7: Serum from WSSV-injected shrimp at 48 hpi.

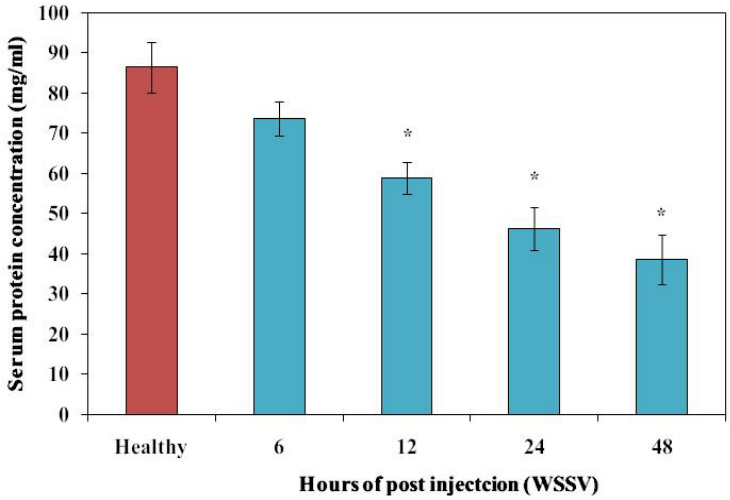

Fig. 2 Analysis of serum protein levels in healthy, sham- and WSSV-injected Litopenaeusvannamei at 6, 12, 24 and 48 hpi.

Each vertical bar represents mean ± SD values of serum protein levels obtained from at least five shrimps. The difference between healthy/control and WSSV- injected shrimps are statistically significant (*p < 0.05) at 12, 24 and 48 hpi.

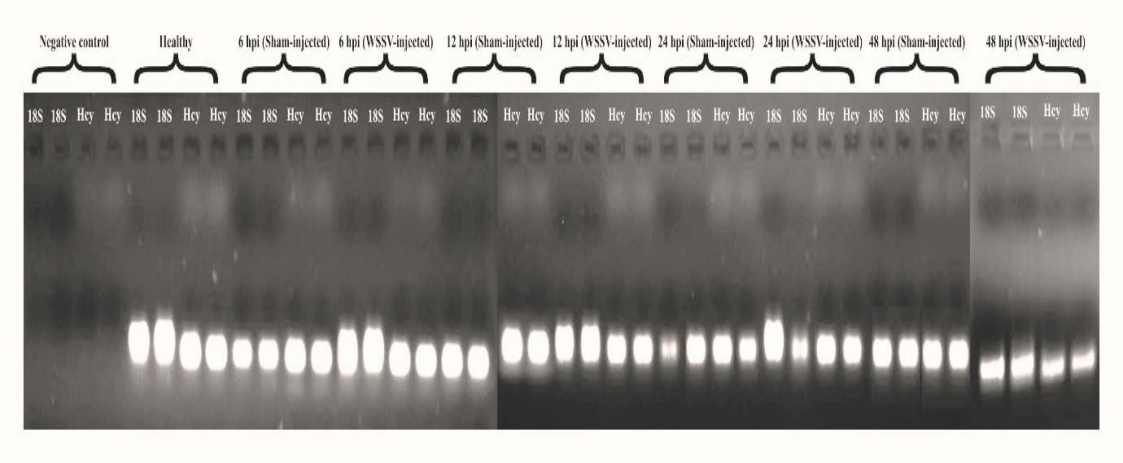

Fig. 3 Analysis of expression of Hemocyaningene I in the hepatopancreas of healthy, sham- and WSSV-injected *L. vannamei*.

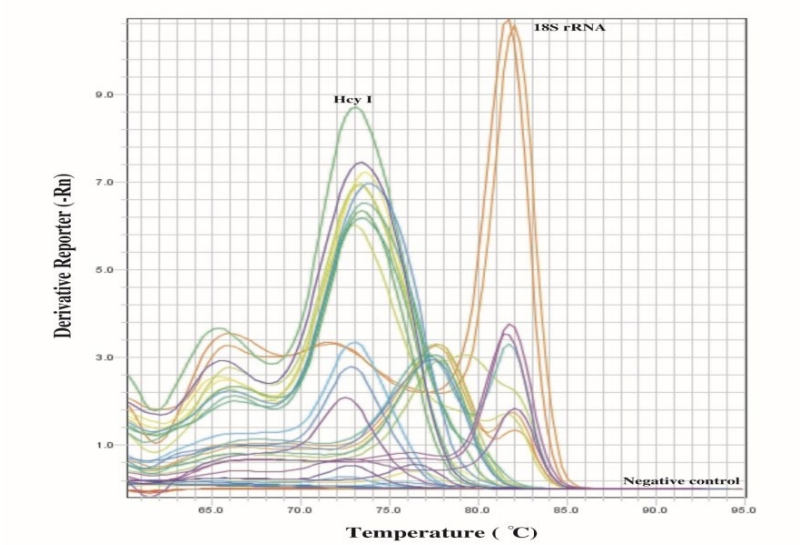

Fig. 4 Analysis of melt curve for the relative quantification of Hemocyanin gene I expression in the hepatopancreas of healthy and WSSV-injected *L. vannamei* at 6, 12, 24 and 48 hpi.

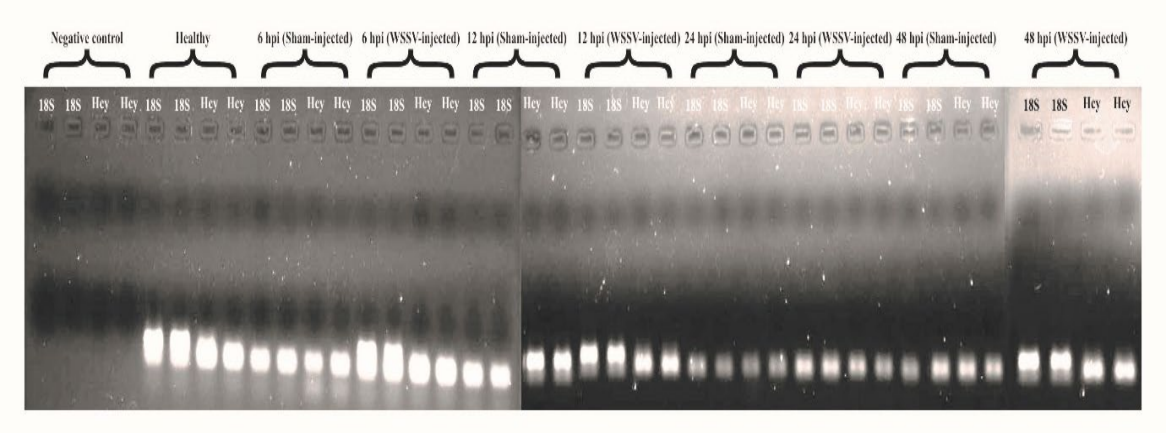

Fig. 5 Analysis of expression of Hemocyanin gene II in the hepatopancreas of healthy, sham- and WSSV-injected *L. vannamei*.

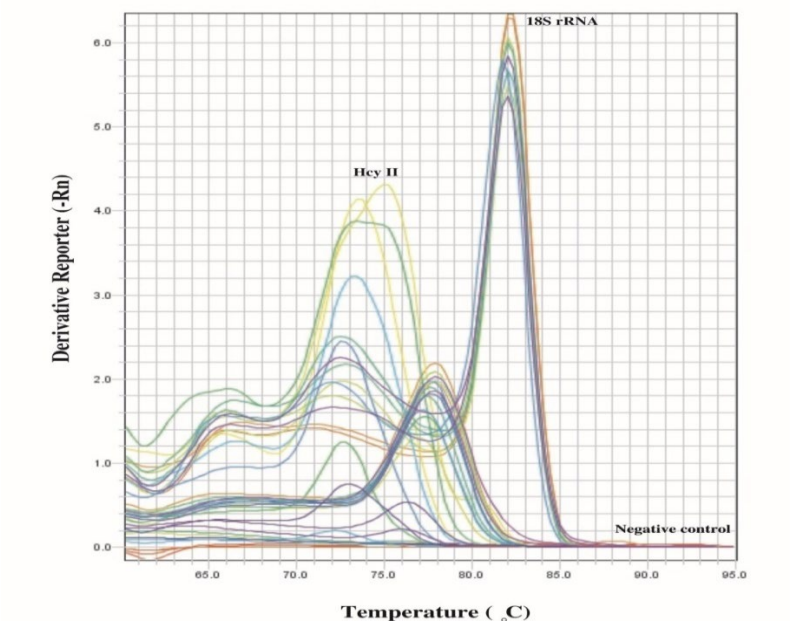

Fig. 6 Analysis of melt curve for the relative quantification of Hemocyanin gene II expression in the hepatopancreas of healthy and WSSV-injected *L. vannamei* at 6, 12, 24 and 48 hpi.

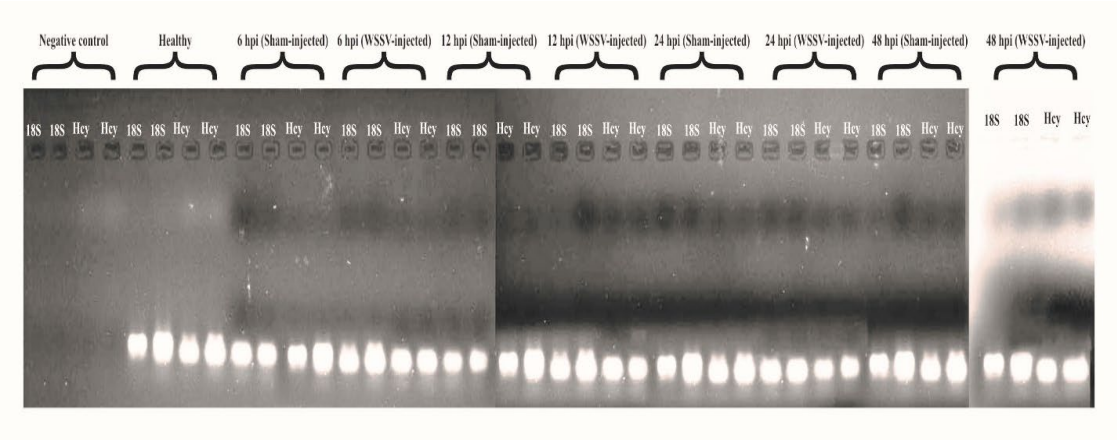

Fig. 7 Analysis of expression of Hemocyanin gene III in the hepatopancreas of healthy, sham- and WSSV-injected *L. Vannamei*

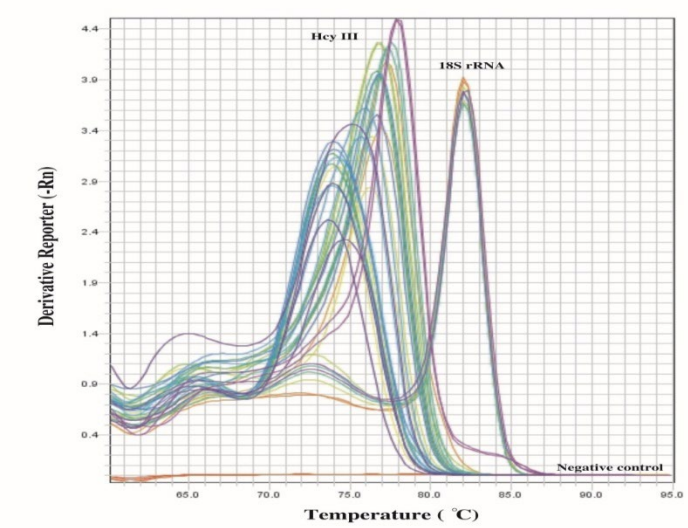

Fig. 8 Analysis of melt curve for the relative quantification of Hemocyanin gene III expression in the hepatopancreas of healthy and WSSV-injected *L. vannamei* at 6, 12, 24 and 48 hpi.

IV. DISCUSSION OF THE STUDY

Litopenaeus vannamei is an exotic species of pacific waters which has been introduced in India replacing the black tiger shrimp which is highly susceptible to WSSV few years back for commercial cultivation. It has increased the shrimp production but could not stop the outbreaks of various diseases especially WSSV-infection. As this shrimp is native to the Pacific region, it has encountered the microbes of the Indian waters with differential responses. Hence, there was a need for a better understanding of the modulations in the immune system of the white leg shrimp upon WSSV-infection (India). In crustaceans, hemocyanin is the predominant protein in the haemolymph, which constitutes 90-95% of the total proteins in systemic circulation (Balaji *et al.,* 1989; Noga, 2000). Besides, it's well known respiratory function, recent studies in crustaceans tend to demonstrate the role of hemocyanin in expression of several defense-related functions including phenol oxidase activity (Decker *et al.,* 2001), haemolytic activity (Zhang *et al.,* 2009), antiviral (Zhang *et al.,* 2004), and antibacterial activities (Lee *et al.,* 2003).

These findings apparently elicited interests to explore unique structural and functional features of hemocyanin in different groups of crustaceans. In accordance with this background, attempts were made in our previous study to isolate hemocyanin from the serum of the white leg shrimp, as a prerequisite to investigate eventually the structure and defense functions of this respiratory protein. In continuation with this in the present study, relative expression of hemocyanin gene in *L. vannamei* upon WSSV-infection has been carried out. In our previous studies the protein profile of serum from *L. vannamei* was examined in native PAGE. When the gel was incubated with BCS that can stain copper-containing proteins (Bruyninckx *et al.,* 1978), four out of eight protein bands were positive for BCS, and the exception being slow moving proximal bands.

These observations clearly indicated that these four BCS-positive bands are copper-containing proteins that presumably include hemocyanin, phenol oxidase, and prophenoloxidase present in the serum *L. vannamei*. As hemocyanins constitute 90-95% of the total proteins in systemic circulation among the bands which were stained positive with BCS, the thick band was confirmed as hemocyanin. This band was found to decrease during WSSV-infection. Similar results were observed in the case of P. monodon in our laboratory. This led us to investigate the alterations in the corresponding gene upon WSSV-infection. This band appears to decrease upon WSSV-injection.

In healthy shrimps, SDS-analysis isolated hemocyanin revealed the presence of three protein bands. Earlier have that hemocyanin has subunits with variability in structure and encoded by distinct genes in an arthropod *Eurypelmacalifornicum.* As a prerequisite, serum protein concentration was determined at different time points of infection. Previous investigators have reported a significant increase (11 or 13%) in haemolymph protein concentration in WSSV-infected P. monodon. This increase was attributed to increase in viral proteins as well as those released from virally infected host tissues (Yoganandhan *et al.,* 2003a; Joseph & Philip, 2007). On the other hand, (Jiang *et al.,* 2010) have reported that the serum protein concentration declined gradually almost resulting in drastic depletion of 50% total serum protein in WSSV-infected *Fenneropenaeuschinensis and Marsupenaeusjaponicus*.

Our previous observations in *P. monodon* has also reported similar pattern of alterations in serum proteins. Drastic decline (44%) of serum proteins was evident towards terminal stage of WSSV infection in *L. vannamei*. Changes in serum protein concentration in crustacean decapods could happen either due to degradation of serum protein, sequestration of serum proteins in tissues or dilution of the haemolymph, which is normally evident in its water content. An analysis of these three factors, would throw light on the reasons behind such contradicting results observed in the literature. In the present study, serum osmolality was found to decrease upon WSSV-infection. This could be due to the decrease in hemocyanin content as it contributes to serum osmolality.

In the present experiment we tried to quantify relative expression on hemocyanin genes (I, II and III) in *L. vannamei* upon WSSV-infection. As a part of this, the specific primers for hemocyanin gene were designed following (Zhao *et al.,* 2016) and relative quantification has been carried out following. As a prerequisite the total RNA has been isolated from the hepatopancreas of the experimental groups of shrimps by RNA isoplus reagent and the purity was checked by running the RNA samples on 1% agarose gel. Immediately first strand of cDNA was synthesized by Revert Aid first strand cDNA synthesis kit and subsequently the cDNA was quantified and checked for the purity through absorbancy values. Analyses of hemocyanin gene (I, II and III) expression in healthy and WSSV-injected shrimps were carried out at 6, 12, 24 and 48 hpi. In these studies, 18S r RNA was used as endogenous control and healthy as reference for the relative quantification. Hemocyanin I gene showed that the relative quantity gradually increases during infection. However, at the end of experiment (48 hpi) a 23867450-fold of increase in expression was observed.

The relative quantity of hemocyanin gene II, during WSSV-infection, increased from 6 hpi (14.9 fold) to 24 hpi (136.9 fold), whereas at 48 hpi the relative quantity was found to decrease by 2-fold when compared to healthy shrimps. On the other hand, hemocyanin gene III showed a moderate increase at 6 and 12 hpi (7.8 fold and 6.2-fold respectively). Same gene at 48 hpi showed an increase of 73951-fold. Electrophoretic analyses of the PCR products on 1% agar and melting curve analyses showed specific multiplication of the target gene in the present study.

Thus, the results showed that all the three hemocyanin genes respond differentially upon WSSV-infection. Previously has

reported the genomic sources of diversity in the hemocyanin gene from *L. vannamei* and showed significant differences in the basal transcriptional levels, and differential immune response after WSSV-challenge, suggesting that hemocyanin possessed molecular diversity and play important roles in the antiviral immunity in shrimps. Another study has reported heterogeneity in hemocyanin subunit of Penaeid shrimp using RNA sequencing.

Our results also showed that the hemocyanin of *L. vannamei* possesses at least three subunits encoded by three distinct genes and the expression of such genes appear to show differential response upon WSSV-infection. Further studies of these three hemocyanin genes and their products in the healthy and WSSV-injected shrimps would provide a better understanding of this gene function. The studies of the hemocyanin gene products and their functional analyses may provide information on specific function of these three genes. Extended studies upon WSSV-infection would reveal clear mechanism of hemocyanin subunits and their role in non-specific immunity. A 45-day feeding trial followed by an acute stress test of low salinity was done to evaluate effects of *Lactobacillus plantarum* on growth performance and anti-stress capability of white shrimp (*Litopenaeusvannamei*) (Zheng *et al.,* 2017).

MicroRNAs (miRNAs) are small non-coding RNAs that regulate diverse cellular processes, including organismal stress response, through posttranscriptional repression of gene transcripts. They are known to have antiviral functions in aquatic crustacean species, but little is known about the role of miRNAs against environmental stress caused by Cu, a common chemical contaminant in aquatic environment. We performed small RNA sequencing to characterize the differentially expressed micro RNAs in Cu exposed shrimp. (Gou *et al.,* 2018)

V. CONCLUSION

The present investigation was carried out to elucidate the effect of WSSV-infection on relative expression of hemocyanin genes (I, II and III) in *Litopenaeus vannamei*, the healthy shrimps were intramuscularly injected with serum from WSSV-infected animal. By the end of 48 hrs after injection all the shrimps showed the symptoms for WSSV-infection. Symptoms like lethargy, reddening of appendages and white spots were observed on the cephalothorax. The shrimps intramuscularly injected with WSSV-infected serum were screened for the presence of WSSV in serum. Serum protein concentration declined gradually during the experimental period. The total RNA was isolated from hepatopancreas in healthy and WSSV-injected shrimps using RNA isoplus reagent. The first strand of cDNA was synthesized using Revert Aid First strand cDNA synthesis kit. The quantity and purity were determined by nanodrop. Relative expression of hemocyanin gene (I, II, and III) was quantified using RTqPCR. Relative expression of hemocyanin gene (I) in WSSV-injected shrimps was found to increase from 6hpi post to 48hpi when compared to healthy shrimps. Relative expression of hemocyanin (II) gene in WSSV-injected shrimps increased from 6hpi to 24 hpi and decreased at 48hpi when compared to healthy shrimps. Relative expression of hemocyanin gene III followed the hemocyanin gene I in relative expression in WSSV-injected shrimps. Analysis of relative expression of all three hemocyanin genes in healthy and WSSV-injected shrimps showed that they respond differentially during WSSV-infection.

REFERENCES

[1] R. G. Adiyodi, "Protein metabolism in relation to reproduction and moulting in the crab, *Paratelphusahydrodromous* (Herbst)". Electrophoretic studies on the mode of utilization of soluble proteins during vitellogenesis. *Indian. J. Exp. Biol.,* Vol. 6, pp. 144-147, 1968.

[2] S. Bailey, A. Melis, K. R. M. Mackey, P. Cardol, G. Finazzi, G. Van Dijken, G. M. Berg, K. Arrigo, J. Shrager and A. Grossman. "Alternative photosynthetic electron flow to oxygen," *Biochim.Biophys.Acta*. Vol. 1777, pp. 269–76, 1994.

[3] R. Balaji, P. Mullainadhan and M. Arumugam, "In vivo binding of exogenous copper to haemolymph fractions of estuarine crab Scylla serrata (Forskal)," *J. Exp. Mar. Biol. Ecol.,* Vol. 128, pp. 241-255, 1989.

[4] J. Bonaventura, and S. C. Wood, "Respiratory pigments: overview," *Am. Zool.,* Vol. 20, pp. 5-6, 1980.

[5] W. J. Bruyninckx, S. Gutteridge and H. S. Mason, "Detection of copper on polyacrylamide gels," *Anal.Biochem.,* Vol. 89, pp. 174-177, 1978.

[6] H. Decker, M. Ryan, E. Jaenicke and N. Terwilliger, "SDS- induced phenoloxidase activity of hemocyanin from Limulus polyphemus, Eurypelmacalifornicum and Cancer magister," *J. Biol. Chem.,* Vol. 276, No. 177, pp. 96-99, 2001.

[7] J. S. Djangmah, "The effects of feeding and starvation on copper in the blood and hepatopancreas, and on the blood proteins of Crangon vulgaris (Fabricius)," *Comp. Biochem. Physiol.,* Vol. 32, pp. 709-718, 1970.

[8] S. Hagner-Holler, A. Schoen, W. Erker, J.H. Marden, R. Rupprecht, H. Decker and T. Burmester, "A respiratory hemocyanin from an insect," *Proc. Nat. Acad. Sci.,* Vol. 101, pp. 871-74, 2004.

[9] E. Hodgson and J. I. Spicer, "Subunit compositions of crustacean hemocyanins are species-specific: evidence from non-decapod species," *Comp. Biochem. Physiol.,* Vol. 128A, pp. 873-88, 2001.

[10] E. C. Horn and M. S. Kerr, "Hemolymph protein and copper concentrations of adult blue crabs (Callinectessapidus Rathbun)," *Biol. Bull.,* Vol. 125, pp. 499-507, 1963.

[11] Hui Guoa, Zhi-cheng Lua, Xiao-wen Zhua, Chun-hua Zhua, Cheng-gui Wanga, Yu-chun Shena and Wei Wangb, "Differential expression of microRNAs in hemocytes from white shrimp Litopenaeus vannamei under copper stress", Vol. 74, pp. 152-161, 2018.

[12] G. Jiang, "Comparison of defense ability against the white spot syndrome virus between Fenneropenaeuschinensis and Marsupenaeus japonicus," *Aquacult. Res.,* Vol.41, pp. 1322-1330, 2010.

[13] A. Joseph and R. Philip, "Acute salinity stress alters the hemolymph metabolic profile of Penaeus monodon and reduces immunocompetence to white spot syndrome virus infection," *Aquaculture*, Vol. 272, pp. 87-97, 2007.

[14] S. Lee, B. Lee and K. Söderhäll, "Processing of an antimicrobial peptide from hemocyanin of the freshwater crayfish, Pacifastacus lenuisculus," *J. Biol. Chem.,* Vol. 278, pp. 7927-33, 2003.

[15] C.A. Leone, "Preliminary observation on intraspecific variation of the levels of total protein in sera of some decapods crustacean," *Science*, Vol. 118, pp. 295-296, 1953.

[16] O. H. Lowry, N. Rosenbrough, A. L. Farr and R. J. Randall, "Protein measurement with the Folin-phenol reagent," *J. Biol. Chem.,* Vol. 193, pp. 265-75, 1951.

[17] M. P. Lynch and K. L. Webb, "Variations in serum constituents of the blue crab, Callinectessapidus: total serum protein," *Comp. Biochem. Physiol.,* Vol. 44A, pp. 1237-1249, 1973.

[18] C. P. Mangum, "Invertebrate blood oxygen carriers," *In 'Handbook of Physiology*, section 13, Comparative Physiology (Ed. W. H. Dantzler), pp. 1097-1131. Oxford University Press, New York, 1997.

[19] J. Mohajeri, M. Afsharnasab, B. Jalali, S. Kakoolaki, M. Sharifrohani and A. Haghighi, "Immunological and histopathological changes in Penaeus semisulcatus challenged with Vibrio harveyi," *Iran J. Fish. Sci.,* Vol. 10, pp. 254-265, 2011.

[20] E. J. Noga, "Hemolymph biomarkers of crustacean health," *In Recent Advance in Marine Biotechnology, Immunobiology and Pathology* (M. Fingerman & R. Nagabhushanam, Eds.), Vol. 5, pp. 125-163, *Science Publishers, Inc.,* New York, 2000.

[21] R. Pais, M. Shekar and I. Karunasagar, "Haem agglutinating activity and electrophoretic pattern of hemolymph serum proteins of Penaeus monodon and Macrobrachiumrosenbergii to white spot syndrome virus injection," *Aquaculture*, Vol. 270, pp. 529-534, 2007.

[22] P. Rameshthangam and P. Ramasamy, "Protein expression in white spot syndrome virus infected Penaeus monodon Fabricius," *Virus Res.,* Vol. 110, pp. 133-141, 2005.

[23] S. Ratanapo and M. Chulavatnatol, "Monodin-induced agglutination of Vibrio vulnificus, a major infective bacterium in black tiger prawn (Penaeus monodon)," *Comp. Biochem. Physiol.,* Vol. 102B, pp. 855-859, 1992.

[24] Y. L. Song, C. I. Yu, T. W. Lien, C. C. Huang and M. N. Lin, "Haemolymph parameters of Pacific white shrimp (*Litopenaeusvannamei*) infected with taura syndrome virus," *Fish Shellfish Immunol.,* Vol. 14, pp. 317-331, 2003.

[25] Xiao-Ting Zheng, Ya-Fei Duan, Hong-Biao Dong and Jia-Song Zhang, "Effects of dietary Lactobacillus plantarum in different treatments on growth performance and immune gene expression of white shrimp Litopenaeus vannamei under normal condition and stress of acute low salinity", *Fish and Shellfish Immunology*, Vol. 1050-4648, No. 17, pp. 25-26, 2017.

[26] K. Yoganandhan, S. Thirupathi and A. S. Sahul Hameed, "A Biochemical, physiological and hematological changes in white spot syndrome virus-infected shrimp, Penaeus indicus," *Aquaculture*, Vol. 221, pp. 1-11, 2003.

[27] S.Zhang, C. Huang and Q. Qin, "Antiviral properties of hemocyanin isolated from shrimp Penaeus monodon," *Antiviral res.,* Vol. 61, pp. 93-99, 2004.

[28] Y. L. Zhang, F. Yan, Z. Hu, X. L. Zhao, S.Y. Min, Z. H. Du, X. Q. Ye and Y. Y. Li, "Hemocyanin from shrimp *Litopenaeus vannamei* shows hemolytic activity," *Fish Shellfish Immunol.,* Vol. 27, pp. 330-335, 2009.

[29] J. Zhao, X. Bao, C. Li, Y. Shen and J. Hou, "Improving monoterpene geraniol production through geranyl diphosphate synthesis regulation in Saccharomyces cerevisiae," *Appl. Microbial. Biotechnol*, Vol. 100, No. 10, pp. 61-71, 2016.