2. REVIEW OF LITERATURE

2.1 Prelude to review

Production from shrimp industry is seriously affected by environmental degradation and infectious and non-infectious diseases. Since vaccination or treatment with antibiotics is not a feasible proposition in shrimp culture, interest is being focused on developing compounds that confer protection and/or enhance immune reactivity to likely pathogens in shrimp. These compounds are thought to act as immunostimulants because of their known effects on the crustacean immune system in vitro (Smith et al., 2003).

For convenience, the literature pertaining to the present study has been reviewed under the following heads:

- Immune system and immune response in shrimp
- Shrimp diseases with reference to vibriosis and white spot syndrome
- Immunomodulators of shrimp immune system
  - Research on immunomodulators in shrimp/ prawn other than Penaeus monodon
  - Research on immunomodulators in Penaeus monodon
  - Environmental factors acting as immunomodulators of shrimp immune system
  - Bacterial biofilm as immunomodulator in aquaculture
2.2 Immune system and immune response in shrimp

An essential component of immunity is the mechanism of surveillance by which an organism can detect the presence of “non-self” molecules. A good non-self recognition system should also stimulate defensive responses, including those mediated by cells. In vertebrates, the immune defense includes adaptive memory, specific immunoglobulins and specialized cells as well as non-specific responses through phagocytic cells and natural killer cells.

Invertebrates do not have antibodies, albeit they possess proteins with domains belonging to the immunoglobulin super family (Lanz Mendoza and Faye, 1996) by which they are able to recognize and destroy invading microorganisms or parasites. Proteins involved in the recognition of cell wall components from microorganisms such as lipopolysaccharide (LPH) and β-1, 3- glucans have been found in invertebrates. However, these proteins are unable to destroy foreign matters and a phagocytic activity is required (Ratcliffe et al., 1985).

Often while describing the components of immune response in crustaceans, a division into humoral and cellular components is used. The humoral factors comprise molecules that act in the defense without direct involvement of cells although many of these factors are originally synthesized and stored in the blood cells. Consequently, the actions with the direct participation of blood cells are understood by the term cellular response (Holmblad and Soderhall, 1999). For evaluation of these humoral and cellular parameters of immune response in shrimp, simplified procedures have been developed (Rodriguez and Le Mouillac, 2000). Several workers have been studying the quantification of different cellular and humoral parameters of the immune response in shrimp (Bachere et al., 1995; Rodriguez et al., 1995; Sung et al., 1996; Vargas – Albores et al., 1996).
2.2.1 Shrimp blood cells

Crustaceans have an open circulatory system with absence of vertebrate red blood cells but analogues of the white blood cells which perform the functions of both exist. In invertebrates, the circulating cells are called as haemocytes which are essential in immunity, performing functions such as phagocytosis, encapsulation and lysis of foreign cells (Smith and Soderhall, 1983; Ratcliffe et al., 1985; Soderhall and Smith, 1986; Johansson and Soderhall, 1989).

2.2.1.1 Types of haemocytes

Crustaceans have three morphologically different haemocyte types: hyaline, semi granular and granular cells (Bauchau, 1980). Granular cells have a large number of secretory granules containing components of prophenoloxidase (proPO) system. Semi-granular cells appear to be the most sensitive ones and react first during an immune response by degranulation. Release of vesicle contents can stimulate the granular cells to degranulate as well (Rodriguez and Le Moullac, 2000).

2.2.1.2. Functions of haemocytes

Functions of haemocytes based on isolated population of cells by different workers are presented as here under:

<table>
<thead>
<tr>
<th>Haemocyte type</th>
<th>Functions in immunity</th>
</tr>
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<tbody>
<tr>
<td>Hyaline cells</td>
<td>Phagocytosis$^1$</td>
</tr>
<tr>
<td>Semi granular cells</td>
<td>Encapsulation$^2$</td>
</tr>
<tr>
<td></td>
<td>Phagocytosis (limited) $^1$</td>
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<tr>
<td></td>
<td>Storage and release of the proPO system$^3$</td>
</tr>
<tr>
<td></td>
<td>Cytotoxicity$^4$</td>
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<tr>
<td>Granular cells</td>
<td>Storage and release of the proPO system$^3$</td>
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<td></td>
<td>Cytotoxicity$^4$</td>
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2.2.1.3 Identification of different types of haemocytes

2.2.1.3.1 Cytochemical techniques

Hose et al. (1987) reported that the acid phosphatase activity was more abundant in semi-granular cells while hyaline cells were distinctively stained by Sudan Black. Sequeira et al. (1996) performed cytochemical staining of haemocyte sub-populations separated by flow cytometry and reported positive peroxidase activity only in granular cells.

2.2.1.3.2 Molecular techniques

An alternative method for cell identification is the use of monoclonal antibodies (MAbs) to find out antigenic markers of different cell types. Using MAbs against different sub-populations of haemocytes separated in a percoll gradient, it was found that hyaline cells share epitopes with semi-granular cells and that an antigen was specifically expressed on semi-granular cells in Penaeus japonicus (Rodriguez et al., 1995).

2.2.2 Clotting and wound healing

Haemolymph coagulation is an essential defense response in crustaceans that prevents loss of haemolymph through breaks in the exoskeleton and dissemination of bacteria throughout the body (Martin et al., 1991). Coagulation is a rapid and powerful process in crustaceans. Clotting is best described in Limulidae, horseshoe crab, where a cascade of proteinases leads to activation of a clotting protein, coagulogen (Kawabata et al., 1996). By the action of transglutaminase
which is stored in the haemocytes and released on activation, covalent cross-links are created so that a clot is formed. Montano-Perez et al. (1999) purified the clotting protein of white shrimp *Penaeus vannamei* by affinity chromatography in a heparin–agarose column. The protein named clotting protein was found to be a lipoglycoprotein composed of two 210-kDa subunits covalently bound by disulfide bridges.

### 2.2.3 Antimicrobial peptides

Antimicrobial peptides and proteins have been well studied in arthropods (Hetru *et al.*, 1994; Iwanaga *et al.*, 1998), where families of antimicrobial molecules have been isolated and characterized. While ample literature is available on different antimicrobial peptides in crab, research in shrimp is scarce. Destoumieux *et al.* (1997) fully characterized the three members of new family of antimicrobial peptides in penaeid shrimp. These peptides, named penaeidins, are the first antimicrobial molecules to be discovered in penaeid shrimp. The penaeidins are 5.5 to 6.6 kDa peptides which combine a proline-rich amino-terminal domain and a carboxyl-domain containing six cysteines engaged in three disulfide bridges. The anti bacterial activity of these penaeidins compared with other effectors of the innate immunity has been extensively reviewed by Bachere *et al.* (2000).

Chiou *et al.* (2007) studied the expression and characterization of *Penaeus monodon* penaeidin in various tissues during early embryonic development and moulting stages using polymerase chain reaction by specific primers. They observed that mo-penaeidin gene consisted of 1348 bp containing one intron (680 bp) and two exons (210 and 458 bp) with an open reading frame of 222 bp which encodes a protein of 74 amino acids including a signal peptide of 19 amino acids. The mo-penaeidin mRNA was detected in various tissues including ovary and mandibular organ. The penaeidin mRNA was found to be present in one
cell to post larva stage with higher level at nauplius I. Also, its expression was significantly higher during intermoult stage.

2.2.4 Phenoloxidase system and melanin formation

The prophenoloxidase (proPO) activating system is one of the best studied immune system in crustaceans with numerous published works on crayfish. The phenoloxidase (PO) is responsible for the melanization process in arthropods where melanin synthesis is involved in the process of sclerotization and wound healing of the cuticle as well as in defense reactions (nodule formation and encapsulation) against invading microorganisms entering the hemocoel (Soderhall, 1982; Ratcliffe et al., 1985; Sugumaran, 1996). The PO enzyme results from the activation of proPO enzyme which is present as an inactive zymogen in haemolymph or cuticle. PO is a bifunctional copper containing enzyme which catalyses o-hydroxylation of monophenols and the oxidation of phenols to quinines (Sugumaran, 1996). Thus, the enzyme is able to convert tyrosine to DOPA, as well as, DOPA to DOPAquinone followed by several intermediate steps that lead to the synthesis of melanin, a brown pigment (Sritunyalucksana and Soderhall, 2000).

The proPO system can be activated by an endogenous activating system and exogenous agents such as lipids, detergents, organic solvents, and microbial elicitors like β-1, 3-glucan, lipopolysaccharide, and peptidoglycan (Ashida and Soderhall, 1984; Ashida and Yamazaki, 1990). In crustaceans, proPO has been demonstrated to be confined to haemocyte granules (Barrett, 1987) and it could also be activated by different chemical and microbial elicitors (Brivio et al., 1992). In addition, Ca\(^{2+}\) is required for the conversion of the proPO-activating enzyme to an active proteinase that transforms proPO to active phenoloxidase.
Biochemical studies on the shrimp proPO system have been carried out in *Farfantepenaeus californiensis* (Johansson and Soderhall, 1985; Brivio *et al*., 1992; Burks and Fuchs, 1995), *Farfantepenaeus paulensis* (Johansson and Soderhall, 1992) and *Penaeus monodon* (Lanz Mendoza *et al*., 1993). proPO has been purified and characterized from haemocytes of *Pacifastacus leniusculus* (Leonard *et al*., 1985) and *F. californiensis* (Burks and Fuchs, 1995) and the molecular masses were 76 and 114 kDa, respectively. *Penaeus monodon* proPO gene was purified and cloned by Sritunyalucksana *et al*. (1999a). The authors reported that shrimp proPO had a 3002 bp cDNA and contained an open reading frame of 2121 bp encoding a putative polypeptide with 688 amino acids and a molecular mass of 78.7 kDa.

### 2.2.5 Pattern recognition proteins

The first immune process in crustaceans is the recognition of invading microorganism which is mediated by the haemocytes and plasmatic proteins (Vargas-Albores *et al*., 1996). Crustaceans do recognize common characteristics present in bacteria and fungus such as lipopolysachharides and β-glucans. There is little information about the molecular mechanisms that mediate recognition; however, in crustaceans, several types of modulator proteins have been described that recognize cell wall components of microorganisms (Bachere, 2000).

Although most research on identification of pattern recognition proteins is focused on crayfish, scanty literature is available with regard to shrimp. In shrimp, both LPS (Vargas-Albores *et al*., 1993; Maheswari *et al*., 1997) and β-glucan (Vargas-Albores *et al*., 1996) binding proteins are present as possible recognition proteins. Vargas-Albores and Yepiz-Plascencia (2000) reported that the mechanism of action of invertebrate recognition protein appeared similar to vertebrate antibodies where, after
reaction with an antigen, the immunoglobulin can activate cellular functions (degranulation and phagocytosis) or plasma complement.

2.2.5.1 β-1, 3 glucan binding protein

β-glucan binding protein (BGBP) and its role in shrimp immune response have been reviewed by Vargas-Albores and Yepiz-Plascencia (2000). Other than two insect species and fresh water crayfish, this protein has been purified in yellow leg shrimp, *P. californiensis* (Vargas-Albores *et al.*, 1996) and white shrimp *P. vannamei* (Vargas-Albores *et al.*, 1997). This protein appeared to be widely distributed among the crustaceans conserving most of its antigenic properties, since a monospecific polyclonal antiserum against *P. leniusculus* BGBP could recognize BGBP from different crustaceans including several shrimp species. In addition, antibodies prepared against purified yellow leg shrimp BGBP clearly detected a 100 kDa protein in plasma from *P. Vannamei* and *P. Stylirostris* (Vargas-Albores *et al.*, 1996).

It was found that BGBP was unable to induce release and activation of the proPO system, but the protein-glucan complex was able to react with the circulating cells and increase the effect of glucans on the proPO system (Borracco *et al.*, 1991; Johansson and Soderhall, 1992; Vargas-Albores, 1995). Thus the recognition proteins are capable of activating cellular activities only after reacting with the microbial carbohydrate (LPS, peptidoglycon and glucans).

2.2.5.2 Lectins

Occurrence, specificity and biological role of crustacean lectins, primarily those of shrimps have been reviewed by Marques and Borracco (1999). Earlier studies have emphasized the possible role of lectins as non-self-recognition molecules in vertebrate and invertebrate immunity (Renwrantz, 1986; Arason, 1996; Matsushita, 1996; Vasta *et al.*, 1999;
Wilson et al., 1999). Due to the fact that lectins have the ability to bind carbohydrate and promote the agglutination of different cells such as bacteria and other invading pathogens, it is reasonable to assume that these molecules may be having a potential role in invertebrate non-self-recognition reactions.

In the penaeid shrimp, *P. monodon*, Ratanapo and Chulavatnatol (1992) reported agglutination of highly pathogenic *Vibrio vulnificus* by a purified lectin called monodin. In the other penaeid, *Penaeus californiensis*, Vargas-Albores et al. (1993) investigated the ability of purified lectin to react with different marine species of *Vibrio*. They demonstrated that the agglutinin of this penaeid was able to react with at least three different *Vibrio* species, *V. vulnificus*, *V. fischeri* and *V. parahaemolyticus*. This reaction was specific and the agglutination of *V. parahaemolyticus* could be inhibited by LPS which suggested that this natural ligand of the penaeid lectin could be one effective sign that trigger the shrimp immune system. In the prawn *P. longirostris*, Fragkiadakis and Stratakis (1995) also reported that purified lectins from the haemolymph that recognised *N*-acyl aminosugars strongly agglutinated formalin-fixed *Pseudomonas aeruginosa* and *E. coli*. The observations of Vazquez et al. (1993, 1996, 1997) on the lectins of the haemolymph of the freshwater prawn *Macrobrachium rosenbergii* are of particular interest. The authors purified and characterized a lectin from the prawn haemolymph and showed that it had the ability to agglutinate several bacteria by recognizing *O*-keto and *O*-methyl containing sugars and *N*-acetyl-sugar in the cell wall. In a later report, Vazquez et al. (1997) demonstrated that the granulocytes of *M. rosenbergii*, in spite of expressing a surface receptor which seemed to correspond to the humoral purified lectin, had the ability to recognize foreign cells in an apparently non-mediated sugar recognition basis.
Ratanapo and Chulavatnatol (1992) reported an elevation of the lectin monodin level in most of *P. monodon* suffering from *V. vulnificus* infection. On the other hand, in the same species, Sritunyalucksana *et al.* (1999b) failed to induce increase in lectin concentration *in vitro* and *in vivo* by using components of microorganism cell wall such as LPS, β-glucans, peptidoglycan and also commercial stimulants.

### 2.2.6 Phagocytosis

Phagocytosis, a most common reaction of cellular defense involves internalization of particles or microorganisms into the cell which later form a digestive vacuole called phagosome. The elimination of phagocytosed particles involves the release of digestive enzymes into the phagosome and generation of reactive oxygen intermediates (ROIs), known as respiratory burst. The first ROI generated during this process is the superoxide anion (O$_2^−$). Subsequent reactions will produce other ROIs such as hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH) and singlet oxygen (O$_2^*$). Hydrogen peroxide can be converted to hypochlorous acid (HOCl) via the myeloperoxidase (MPO) - H$_2$O$_2$-CL system, forming a potent antibacterial system (Bayne, 1990).

In penaeid shrimp, most studies on phagocytosis have been performed through observations of clearance of injected bacteria or particulate materials (Martin *et al.*, 1993). Most studies on ROIs generation in invertebrates have been conducted in mollusks (Bachere *et al.*, 1991; Pipe, 1992; Anderson, 1994). Quantitative procedures have been applied for shrimp ROIs generation such as nitroblue tetrazolium (NBT) reduction technique for the measurement of intracellular O$_2^−$ and the reduction of ferricytochrome C for extracellular O$_2^−$ (Rodriguez and Le Moullac, 2000).
Song and Hsieh (1994) described for the first time the oxidative metabolism in *P. monodon*. They measured O$_2^-$ using NBT reduction technique and H$_2$O$_2$ by HRP dependent oxidation of phenol red. Bachere *et al.* (1995) demonstrated the existence of respiratory burst in *P. japonicus* induced by zymosan. Le Moullac and Haffner (2000) emphasized the importance of respiratory burst in *P. vannamei* and its value as biomarker of environmental disturbances.

Deachamag *et al.* (2007) studied the expression of a phagocytosis activating protein (PAP) gene in immunized *P. monodon*. It was reported that immunostimulation with inactivated *Vibrio harveyi* induced the PAP gene which is a ribosomal protein L26 (RPL26) gene and facilitated the protective defense against WSSV infection. The expression level of the PAP gene served as an indicator of the immune response in cultured shrimp.

### 2.2.7 Plasma protein

One of the important functions of haemolymph in crustaceans is to transport molecules such as the respiratory protein (haemocyanin) which is the most abundant molecule of the haemolymph (60 to 95 per cent of total protein) followed by the clotting protein and other humoral components (Djangmah, 1970). In shrimp, concentration of plasma proteins is related to moult cycle. Chen and Cheng (1993) observed that in *P. japonicus*, the plasma protein levels were lower during post moult as opposed to higher levels found in early pre-moult.

### 2.3 Shrimp diseases with reference to vibriosis and white spot syndrome

Among the diseases of shrimp, the diseases caused by viral, bacterial, fungal, protozoan and rickettsial etiologies have gained considerable importance (Lightner, 1988; Brock and Lightner, 1990).
During the last decade, it has been reported that infectious diseases caused by virus followed by bacteria have caused massive mortalities in shrimp culture around the globe (Flegel, 2006).

### 2.3.1 Vibriosis

*Vibrio* spp. is a Gram negative, oxidase positive and motile organism. Various species of *Vibrio* like *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. dansela*, *V. harveyi*, *V. anguillarum*, *V. nereis* and *V. fluvialis* have been described as the principal pathogenic species that affect penaeid shrimp (Lightner et al., 1993). This bacterium is known to take advantage of ecological changes in culture system and to cause periodic diseases in shrimp (Skjermo and Vadstein, 1999). The effect and severity of disease in shrimp are mainly related to the type of *Vibrio* spp., level of infection, water quality, feed and shrimp quality at the time of stocking into pond (Lightner et al. 1983). Yasuda and Kitao, (1983) observed low growth rate of shrimp larvae at protozoal stage when *Vibrio* were present at higher concentration (10$^7$ cfu/g) in water and shrimp gut. Mortalities in *P. monodon* and *P. merguiensis* larvae have been observed in Indonesia, Thailand, Philippines and other countries (Johnson, 1994). The mortality reported ranged from insignificant to 100 per cent, particularly in post larvae and juvenile shrimps. In juvenile and adult shrimps, diseases due to *Vibrio* are commonly known as Sea gull syndrome (Lightner, 1983), Red disease syndrome (Alapide-Tendencia and Dureza, 1997), Tea brown gill syndrome (Ruangpan et al., 1999) and Syndrome-93 (Costa et al., 1998). The species primarily involved were *V. harveyi*, *V. fluvialis*, *V. parahaemolyticus* and *V. penaeicida*. In larvae and postlarvae, vibriosis is classified as oral/enteric vibriosis and appendage/cuticular vibriosis.
2.3.2 White spot syndrome

White spot syndrome is a disease caused by white spot syndrome virus (WSSV) in shrimps (Lo et al., 1997). WSSV infection is characterized by gross lesions of white spots of various sizes embedded in the cuticle at the later stages of infection. These lesions were first reported from an outbreak that occurred in *P. japonicus* in Japan in 1993. The causative agent was a new bacilliform virus which is now called white spot syndrome virus by general consensus (Lightner and Redman, 1998). White spot syndrome virus was originally called as baculovirus based on its cylindrical morphology and histological lesions that resembled “non-occluded” baculoviruses (Wongteerasupaya et al., 1995). It is now known that WSSV is a tailed, rod shaped, double stranded DNA virus with a very large circular genome in the order of 300 kbp. Since the genome had no significant homology to any known virus (Yang et al., 2001), a new viral family (Nimaviridae) and genus (Whispovirus) were created to accommodate it (Mayo, 2002).

2.4 Immunomodulators of shrimp immune system

Immunostimulants, in general increase resistance to infectious diseases by enhancing non-specific defense mechanisms. Since there is no memory component involved, the response is likely to be of short duration (Sakai, 1999).

The disease out breaks pose a continual threat to the existence of any shellfish farm or hatchery. Once an infection occurs it can prove devastating to the entire stock. To some extent good husbandry practices may help but additional forms of protection are necessary to prevent epidemics. Application of antibiotics or other chemicals is undesirable due to heavy cost involved and also risk of contamination of both environment and final product (Grant and Briggs, 1998).
application of antibiotics in the long term may lead to spread of drug resistant pathogens (Smith et al., 1994). Hence, there is a need to maximize the immunocompetence of the stock while minimizing the use of therapeutic agents (Bachere et al., 1995). As of now, there is no evidence that crustaceans share with vertebrates, clonally derived subsets of cells that permit specific, adaptive and ‘memory-based’ immunity that is the basis for conventional vaccination regimen. Further, crustaceans do not appear to possess immunoglobulin molecules and a complete complement system or there is nothing to suggest that they demonstrate the rearrangement of genes that underpin the generation of diversity within the vertebrate immune system. Hence, immunostimulants must act on the innate immune system of crustaceans and therefore, it can be presumed that these immunostimulants can boost the non-specific defense system to improve surveillance and reaction towards potential non-self threats (Smith et al., 2003).

### 2.4.1 Research on immunomodulators in shrimp or prawn other than *P. monodon*

To examine the potency of oral administration of peptidoglycan (PG) derived from *Bifidobacterium thermophilum*, Itami et al. (1998) administered PG to kuruma shrimp (*P. japonicus*) through diet at 0.2 mg/kg body weight/day for 7 consecutive days, alternated with 7 days without PG throughout a 95-day test period. After sampling the shrimp on Day 65 and 95, they were challenged with *Vibrio penaeicida* and WSSV individually. The survival rate of PG-fed group was significantly higher than the control in both the challenge studies. Further, Phagocytic index of PG-fed shrimps was higher than that of the control.

Enhancement of resistance against vibriosis in juvenile *P. vannamei* by supplementation of diets with different yeast products was evaluated
by Scholz et al. (1999). The shrimps were reared on five different experimental diets containing *Saccharomyces cerevisiae* (1 per cent), β-glucan extracted from *S. cerevisiae* (0.1 per cent), *Phaffia rhodozyma* (1 per cent), experimental yeast HPPR1 (1 per cent) and a control diet. Twenty-four hours after immersing the shrimps in a viable cell suspension of *V. harveyi*, the shrimps which were fed with *S. cerevisiae*, *P. rhodozyma*, and HPPR1 and control diet had effectively cleared the bacteria from the haemolymph while the shrimps fed with glucan diet showed elevated bacterial count. Determination of phenoloxidase activity of shrimps showed a significant difference among the five treatments with phenoloxidase activity for the *Phaffia*-treated shrimps being significantly lower than any other diets except the β-glucan diet.

Takahashi et al. (2000) studied the enhancement of disease resistance against Penaeid acute viraemia and induction of virus-inactivating activity in haemolymph of *P. japonicus*, by oral administration of *Pantoea agglomerans* lipopolysaccharide (LPS) and observed that the oral administration of LPS increased the phagocytic and PO activity of shrimp haemocytes. Also, virus-inactivating activity was induced in the haemolymph which might play an important role in controlling the viral infection in shrimp.

The immunomodulatory action of superoxide dismutase (SOD) and its possible use as an indicator of immune response in American white shrimp (*Litopenaeus vannamei*) was studied by Campa-Courdova et al. (2002a). The SOD activity in haemocytes was quantified to evaluate whether β-glucan and sulfated polysaccharide induced immunostimulatory activity. The haemocytes showed increased levels of SOD activity and decreased total haemocyte count within 24 h after administration of immunostimulants. The total haemocyte count and total soluble haemolymph protein increased over normal values after 48-
120 h. It was concluded that the single immunostimulation with $\beta$-glucan and sulfated polysaccharide was sufficient to generate an increase in the antioxidant activity of _L. vannamei_ SOD.

Lopez _et al._ (2003) designed a study to determine the effect of dietary $\beta$ 1-3 glucan (BG) and a mega dose of vitamin C on the immunological system in _L. vannamei_ juveniles. The authors recorded higher blood protein, total blood cells, granular cells and PO activity in shrimp fed with vitamin C as compared to the remaining treatments.

Pascual _et al._ (2004a) fed shrimps with a high (HCHO: 44 per cent) or a low (LCHO: 3 per cent) carbohydrate diet for 55 d to _L. vannamei_ juveniles. The authors found a direct relation between dietary CHO and lactate, protein and haemocyte levels indicating that dietary CHO was used for protein synthesis via transamination pathways in wild shrimp and in farmed shrimp these parameters were inversely proportional to dietary CHO level indicating that the capacity to synthesize protein from dietary CHO was repressed in cultured shrimp.

In a study designed to evaluate the effect of dietary protein level on survival and immunological condition of _L. vannamei_ juveniles, Pascual _et al._ (2004b) observed not only a reduction of haemocytes in shrimp fed sub-optimal dietary protein levels but also reduction in zymogens contained in haemocytes, i.e., prophenoloxidase (ProPO) system, peneidins and their activities (phagocytosis, coagulation).

In an experiment to study the effect of replacement of fish meal by meat and bone meal and poultry by-product meal in diets on the growth and immune response of _Macrobrachium nipponense_, Yang _et al._ (2004) observed no significant difference in immunological parameters including total haemocyte count, PO activity and respiratory burst while the values
for all the immunological parameters studied in the control group were significantly higher than those in replacement group.

Maggioni et al. (2004), in a study to examine the modulation of some hemato-immunological parameters in female *L. vannamei* submitted to unilateral eyestalk ablation and whose diet was supplemented with high doses of vitamin C as a form of immunostimulation, observed absence of significant changes in the hemato-immunological parameters suggesting the existence of a compensatory mechanism induced by the non-ablated eyestalk.

Cheng et al. (2005b) observed that the total haemocyte count, PO activity, respiratory burst and phagocytic activity and clearance efficacy of the shrimp *L. vannamei* increased significantly when shrimps were administered sodium alginate at different concentrations with feed for five months. The survival of the shrimp after challenging them with *V. alginolyticus* was also significantly higher in sodium alginate fed group.

Protective effect of chitin and chitosan against *V.alginolyticus* in white shrimp, *L. vannamei* was studied by Wang and Chen (2005), after injecting the shrimps with either chitin or chitosan at different concentrations. It was observed that the survival of shrimps that received chitin or chitosan was significantly higher than that of control shrimp at the termination of the experiment. Also, it was found that shrimp which received chitin at 6 µg/g or chitosan at 2 and 4 µg/g had higher total haemocyte count, respiratory burst, PO activity and phagocytic activity against *V. alginolyticus* indicating that chitin and chitosan increased the immune ability and resistance to *V. alginolyticus* infection in *L. vannamei*.

The immunostimulatory effects of hot water extracts of *Gracilaria tenuistipitata* (Hou and Chen, 2005) and *Gelidium amanarasi* (Fu et al.,
2006) on the white shrimp *L. vannamei* and their resistance against *V. alginolyticus* were investigated. In these studies, total haemocyte count, PO activity, respiratory burst, phagocytic activity and clearance efficacy to *V. alginolyticus* were examined after shrimps were individually injected with hot water extracts of *G. tenuistipitata* and *G. amanasii* at different concentrations. At all the dosages, total hemocyte count, phenoloxidase activity and respiratory burst increased after two days while phagocytic activity and clearance efficacy increased after one day of injection with *G. tenuistipitata*. Also, the survival of shrimps challenged with *V. alginolyticus* was higher in shrimps that received *G. tenuistipitata* or *G. amanasii*. The above investigations revealed that *L. vannamei* that received hot-water extracts of *G. tenuistipitata* or *G. amanasii* had enhanced immunity and increased resistance against *V. alginolyticus* and hence, the hot-water extract of both the algae could be used as immunostimulants for *L. vannamei*.

The total haemocyte count, PO activity, respiratory burst, phagocytic activity and clearance efficacy to *V. alginolyticus* were evaluated after injecting the white shrimp *L. vannamei* with dopamine or noradrenaline at $10^{-8}$, $10^{-7}$, and $10^{-6}$ mol/shrimp (Cheng et al., 2005c; Cheng et al., 2006a). The results revealed that shrimps which received dopamine or noradrenaline had increased susceptibility to *V. alginolyticus* infection. Also, the values for different immunological parameters declined in those shrimp that received dopamine or noradrenaline.

In a study to assess the effect of *Sargassum fusiforme* polysaccharide extracts on vibriosis resistance and immune activity of the shrimp, *Fenneropenaeus chinensis*, Huang et al. (2006) observed that the oral administration of *Sargassum fusiforme* polysaccharide extracts at an optimal level of 0.5 and 1.0 per cent for 14 d effectively improved vibriosis resistance and enhanced immune activity of shrimps in general.
Mercier et al. (2006) subjected juvenile shrimp, *L. vannamei* reared in either outdoor concrete tanks or indoor plastic tanks to a repeated stress induced by daily handling for 4 weeks and compared the immune response (total haemocyte count, superoxide anion production, and superoxide dismutase activity) with unstressed shrimp. The authors observed no significant differences between stressed and unstressed shrimps raised in either experimental system, suggesting that repeated stress did not affect the immune response.

Sajeevan et al. (2006) studied the immunostimulatory effect of marine yeast *Candida sake* S165 in *Fenneropenaeus indicus* by feeding the shrimps for 28 d with varying biomass concentrations of the yeast and observed that 10 per cent *C. sake* in the diet was found to elicit an optimum immune response in shrimps in general.

Wang et al. (2006) demonstrated that supplementation of ascorbic acid in enriched live food enhanced the anti-oxidant capacity of shrimp, increasing its defense system that may fight against environmental stress leading to reduced ammonia toxicity.

Cheng et al. (2006b) observed that the susceptibility of *M. japonicus* to *V. alginolyticus* correlated with reductions in immune functions like decrease in total haemocyte count, reduction in hyaline cells, PO activity, phagocytic activity and clearance efficacy to *V. alginolyticus* when shrimps were exposed to sulphide at 575 µg/litre or more.

**2.4.2 Research on immunomodulators in *Penaeus monodon***

In an experiment to study the *In vitro* effect of microbial cell wall components peptidoglycan (PG), lipopolysaccharide (LPS) and laminarin, Sritunyalucksana et al. (1999b) observed increased PO activity in laminarin fed shrimp and decreased antibacterial activity in LPS fed
shrimp. The authors also suggested involvement of LPS in mechanisms for both clotting and for antibacterial activity.

Cheng et al. (2000) evaluated the immunomodulatory effects of dietary β-1,3-glucan derived from Schizophyllum commune, in the brooders of P. monodon and observed enhanced phagocytic activity, cell adhesion and superoxide anion production in shrimps.

Immunity enhancement in the shrimp, P. monodon by a probiont Bacillus spp. was studied by Rengpipat et al. (2000). Survival and growth of shrimps fed probiont in 290 d culture trials were better when compared with control shrimps. The phagocytic activity, PO and antibacterial activity were found to be increased by feeding Bacillus S11. Further, survival of shrimps infected with pathogenic V. haeveyi was higher in probiont bacteria fed shrimps. The results documented that Bacillus S11 provided disease protection by activating both cellular and humoral immune defense functions as well as providing competitive exclusion in the shrimp’s gut.

Effect of dietary copper on the non-specific immune responses of juvenile P. monodon was investigated by Lee and Shiau (2002). The results revealed that shrimps fed diets supplemented with 10 and 20 mg Cu/Kg had better weight gain, increased feed and protein efficiency, increased total haemocyte count and intracellular superoxide anion production than those fed unsupplemented control diet.

Lee and Shiau (2003) reported that increase in dietary vitamin C levels in the diet improved the respiratory burst response and prevented tissue copper accumulation in P. monodon fed with high dietary copper.

Preparation of spent brewer’s yeast β-glucans with a potential application as an immunostimulant for P. monodon was investigated by Suphantharika et al. (2003). In vitro, they observed enhanced
phenoloxidase activity in the treated shrimp haemolymph when compared to controls without glucan. Also, in vivo, an oral administration of 0.2 per cent glucan in diets for three days revealed increase in the PO activity of the shrimps.

Azad et al. (2005) studied the routes of immunostimulation vis-a-vis survival and growth of *P. monodon* post larvae and suggested that the booster dose of immunostimulation, in general, was advantageous in inducing growth and protective response in shrimps. Also, they indicated that in-feed route of administration was more practical as well as productive.

Supamattaya et al. (2005) studied the effect of commercially available *Dunaliella* extract on growth performance, health condition, immune response and disease resistance in *P. monodon*. The authors observed higher resistance to WSSV infection and better tolerance to stress induced by low dissolved oxygen condition when *Dunaliella* extract was fed at a dose of 300 mg/ kg feed. However, the shrimps fed 125–300 mg of *Dunaliella* extract/kg diet for 8 weeks showed higher weight gain and survival compared to the control but there was no significant difference in total haemocyte count and phenoloxidase activity among the treatment groups.

Immunostimulatory effect of methanolic extracts of selected Indian immunostimulant herbs (*Cyanodon dactylon, Aegle marmelos, Tinospora cordifolia, Picrorhiza kurooa* and *Eclipta alba*) against WSSV infection in *P. monodon* with reference to haematological, biochemical and immunological changes was studied by Citarasu et al. (2006). Among the different concentrations of herbal immunostimulant supplemented diets, the shrimps fed on diet containing 800 mg /kg of herbal extract had better survival and reduction in the viral load. Also, better values of haematological, biochemical and immunological parameters were
observed in shrimps treated with herbal immunostimulants. The findings of the study revealed that the application of herbal immunostimulants was effective against shrimp viral pathogens.

Shiau and Jiang (2006) conducted an 8-week feeding trial to determine the dietary zinc requirement and its effect on the non-specific immune responses of juvenile *P. monodon* by providing 7, 17.5, 28, 35, 48, 57, 87 and 127 mg Zinc/kg diet. Shrimp fed diets supplemented with ≥35 mg Zn/kg had greater weight gain than those fed diets with ≤17.5 mg Zn/kg. Both intracellular superoxide anion production ratios and total haemocyte count were better in shrimp fed diets with 35 and 48 mg Zn/kg diet. The immune indicators suggested that an adequate dietary Zn concentration for better nonspecific immune responses in *P. monodon* was about 35–48 mg Zn/kg diet.

Chang *et al.* (2007) investigated the adverse effects of dopamine on the immunity in *P. monodon* by measuring the total haemocyte count, differential haemocyte count, PO activity, respiratory burst, superoxide dismutase activity, phagocytic activity and clearance efficiency to the pathogen *Photobacterium damsela* and concluded that stress-inducing dopamine suppressed the immune system, which in turn increased the susceptibility of *P. monodon* to *P. damsela*.

### 2.4.3 Environmental factors acting as immunomodulators of shrimp immune system

Cultured shrimp are subjected to climatic changes and changes due to rearing practices that influence the physico-chemical quality of water. Physico-chemical changes of sea water affect the metabolism, growth, moulting and survival that can influence the immune system (Le Moullac and Haffner, 2000). Most research work related to fluctuations in natural environment and immune responses is carried out in the crab,
Carcinus maenas. However, scanty literature is available on the effect of environmental insults on the immune response in shrimp.

It is reported that low oxygen tension hampers the metabolic performances in shrimp and can reduce growth and moulting frequency (Allan and Maguire, 1991) and cause mortality (Madenjian et al., 1987). Crustaceans show several adaptation responses to hypoxia such as reduction of metabolic rate (Hill et al., 1991) and change in osmotic pressure of the haemolymph (Charmantier et al., 1994). Decrease in dissolved oxygen is a common hazard in shrimp culture (Jiang et al., 2005).

The immune response of P. stylirostris exposed to severe hypoxia was measured in terms of total haemocyte count, differential haemocyte count, PO activity and respiratory burst (Le Moullac et al., 1998). Hypoxia induced a decrease of total haemocyte count which was due to a decrease in semi granular cells and hyaline cells. On the other hand, increased PO activity was related to a reduction of plasma inhibitors regulating the proPO system. There was also a decrease in the total NBT staining, although the activity per cell did not change.

In P. monodon, the phagocytic activity of haemocyte was less efficient in oxygen depleted shrimp (Direkbusarakom and Danayadol, 1998). The average clearance efficiency of oxygen-depleted shrimp was approximately 50 per cent less than that in control shrimp. Le Moullac et al. (1998) measured in vitro the ability of haemocytes after the stress, whereas Direkbusarakom and Danayadol (1998) stimulated first in vivo the defenses by injecting the shrimp with a yeast suspension, and in these conditions, plasmatic recognition factors were involved in phagocytosis. The decrease in total haemocyte count in P. stylirostris and phagocytosis in P. monodon was attributed to low oxygen level in the
pond water which caused an increased susceptibility to infectious diseases.

Jiang et al. (2005), in an experiment to study the effect of dissolved oxygen on immune parameters of the white shrimp, *L. vannamei*, observed decreased THC /antibacterial activity and increased PO activity in shrimps exposed to 3.3 and 2.0 mg O₂/litre when compared to control shrimps exposed to 7.5 mg O₂/litre.

Li et al. (2006) studied the effects of dissolved oxygen concentration and stocking density on growth and non-specific immunity factors in Chinese shrimp, *Fenneropenaeus chinensis*. The results revealed that dissolved oxygen concentration was one of the key factors affecting shrimps through influencing activities of non-specific immunity, while the stocking density affected the growth performance of shrimp mainly by influencing the activities of enzymes and the interactive effects of dissolved oxygen concentration and stocking density played a crucial role in the production of shrimp.

Water temperature is probably the most important environmental variable because it directly affects metabolism, oxygen consumption, growth, moulting and survival (Chen et al., 1995; Henning and Andreatta, 1998). Temperature has a direct effect on other environmental parameters such as salinity and oxygenation of the water. In the brown shrimp, *P.californiensis*, a temperature increase from 18°C to 32°C affected haemolymph parameters, showing a decrease in total haemolymph proPO at 32°C and an increase of plasmatic protein at 28°C and 32°C (Vargas-Albores et al., 1998).

Cheng et al. (2005a) studied the effect of water temperature on the immune response of *L. vannamei* and susceptibility to *V.alginolyticus* and concluded that transfer of shrimp from 27 or 28°C to higher
temperatures (32 and 34°C) reduced their immune capability and resistance to *V. alginolyticus* infection.

The immune response of *P. monodon* and its susceptibility *Photobacterium damselae* under temperature stress was investigated by Wang and Chen (2006). The authors concluded that transfer of *P. monodon* from 26°C to 22°C and 34°C reduced their resistance against *Photobacterium damselae* infection.

In another experiment to understand how stress induced by extreme temperature modulates the immunological behaviour of *Litopenaeus setiferus* males, Pascual *et al.* (2003) used some immune responses as indicators of stress and reported that high temperature caused a reduction in haemocyte proPO activity.

In *P. stylirostris*, the effect of temperature drop from 27°C to 18°C during 24 h on total haemocyte count and PO activity was studied (Le Moullac and Haffner, 2000). It was observed that in shrimp exposed to low temperature, THC dropped by 40 per cent whereas, PO activity increased significantly. However, adaptation phenomena were observed since in the cold season in New Caledonia, when the temperature was around 20°C, the total haemocyte count in *P. stylirostris* was as elevated as in the hot season (Le Moullac and Haffner, 2000).

It is suggested that maximum growth of an organism occurs in an isoosmotic media, since the animal would be expending the minimal amount of energy in osmotic regulation. However, salinity itself has little effect on the metabolic rate of euryhaline shrimp, indicating that the energy required for osmotic regulation may be relatively small. On the other hand, under unhealthy conditions such as viral infections, the stress provoked by high salinity further augments growth retardation produced by the infection (Bray *et al.*, 1990).
The effect of salinity on plasma protein concentration and total haemocytic proPO has been studied by Vargas-Albores et al. (1998) in *P. californiensis*. In this study, juvenile shrimps were acclimatized for 20 d at different salinities (28, 32, 36, 40 and 4‰ at 25°C). Total protein levels were not affected, but total proPO increased as salinity increased.

In order to look for technically simple, rapid and low cost stress indicators, Perazzolo et al. (2002) evaluated some haematological parameters in the shrimp *Farfantepenaeus paulensis* submitted to environmental and physiological stress like low salinity, unilateral eyestalk ablation in females and spermatophore extirpation in males. Among the assessed hemato-immunological parameters, the total haemocyte counts and the total serum protein concentration were found to be the most promising parameters to indicate shrimp stress status.

Effects of mercury on the immune functions have been studied in the fresh water prawn, *Machrobrachium idae* (Victor et al., 1990). The prawns exposed to 1 µg/litre of mercuric chloride over a 30 d period exhibited hyperplastic gill lamellae engorged with haemocytes. It was suggested that the metal could affect haematopoiesis since mercury at a concentration of 50 µg/litre suppressed the circadian rhythmicity of haemocyte numbers.

Effects of short term (96 h) exposure to dissolved heavy metals (mercury, cadmium, lead, copper, chromium and zinc) on the number of circulating haemocytes in the shrimp, *Palaemon elegans* was investigated by Lorenzon et al. (2001). Changes in haemocyte counts were determined in relation to time of exposure and metal concentration. It was found that immersion in artificial sea water containing these heavy metals caused a decrease in the haemocyte count during the first 8 h of exposure, although the haemocyte counts returned to initial levels over
the following 16 h of immersion. The greatest decrease in haemocyte numbers was induced by lead, followed by zinc, mercury, chromium, copper and cadmium.

Effect of copper sulfate on the immune response and susceptibility to *V. alginolyticus* in *L. vannamei* was studied by Yeh *et al.*, (2004). Shrimps were challenged with *V. alginolyticus* and then placed in water containing different concentrations of copper. Shrimps exposed to copper for 24 h showed decreased THC, PO activity, phagocytic activity and clearance efficiency as well as increased mortality due to *V. alginolyticus* infection.

Short term (96 h) toxic effects of copper and cadmium at sub-lethal concentrations on the total haemocyte count and serum phenoloxidase activity in *Fenneropenaeus indicus* were investigated in relation to time of exposure and concentration of the metals used (Sharma *et al.*, 2005). It was observed that decrease in haemocyte count and PO activity values in shrimps exposed to metals were rapid and transient. Also, the rapid development of hemocytopenia and decreased PO activity was more conspicuous in case of shrimp exposed to cadmium than those exposed to copper.

Ammonia is known to be very toxic to aquatic animals and can cause impairment in numerous organs (Colt and Armstrong, 1981). In the intensive culture system, ammonia is the commonest toxicant resulting from excretion by cultured animals and ammonification of unconsumed feed (Le Moullac and Haffner, 2000).

An experiment to determine the dose-response effect of ammonia was carried out on shrimp immune response including the study of expression of the proPO and peroxinectin genes in *P. stylirostris* (Le Moullac and Haffner, 2000). The treatment resulted in reduction in the
amount of haemocytes by 15 per cent at 1.5 mg/litre and 50 per cent at 3.0 mg/litre. Concurrently, the amount of transprict encoding proPO and peroxinectin decreased by 60% and 50%, respectively in response to stress.

Immune response of *L. vannamei* and its susceptibility to *V. alginolyticus* under ammonia stress was studied by Liu and Chen (2004). Among the different immune parameters studied, no difference in total haemocyte count was observed among shrimps at different ammonia-N concentrations. PO activity however, decreased when the shrimps were exposed to 5.24 mg/litre ammonia-N and greater after 7 days. It was concluded that ammonia in water caused a depression in the immune response and an increase in mortality of *L. vannamei* from *V. alginolyticus* infection.

In an experiment to study the susceptibility of *L. vannamei* to *V. alginolyticus* under nitrite stress, Tseng and Chen (2004) challenged the shrimp with *V. alginolyticus* and then placed in water containing different concentrations of nitrite. It was observed that nitrite in water caused a depression in the immune ability of *L. vannamei* to *V. alginolyticus* infection together with an increase in super oxide anion production.

In an experiment to examine the effects of harbour dredge spoils on the immune capability of common shrimp, *Crangon crangon*, Smith *et al.* (1995b) observed that the immune capability was adversely affected in shrimps exposed to harbour dredge spoils as indicated by elevation in recoverable haemolymph volume, reduction in total haemocyte count and reduced blood cell phenoloxidase activity.

Propiconzole, a fungicide, injection in shrimp *P. vannamei* induced an increase in respiratory burst on Day 6 following injection where as, on Day 13, a significant dose-dependant decrease of the respiratory burst to
the injected amount of Propiconzole, was observed (Le Moullac and Haffner, 2000).

Immune response of *L. vannamei* and its susceptibility to *Vibrio* infection in relation to moult cycle was studied by Liu et al. (2004). It was observed that THC, PO activity, respiratory burst and clearance efficiency were highest in intermoult but lower at post moult stages. Also, the mortality of shrimps injected with *V. alginolyticus* was significantly higher in shrimps at postmoult stage than those at intermoult stage. The authors concluded that *L. vannamei* showed a decrease in resistance to infection due to decrease in immunological values at post moult stage when compared to other stages.

In an attempt to know how starvation level modulates catabolism and its effects on the immune response, Pascual et al. (2006) studied juvenile *L. vannamei* that had been starved for varying period after being conditioned on diet containing either maintenance or optimal dietary protein levels and observed a reduction in all the physiological and immunological indicators with starvation. It was suggested that shrimps with good nutritional condition could tolerate starvation until 14 d without modifying the evaluated immune responses.

### 2.4.4 Bacterial biofilm as immunomodulator in aquaculture

Natural bacterial populations tend to occur as assemblages enmeshed in a polymeric glycocalyx matrix called biofilm to take advantage of the nutrient concentrating effect and to gain protection against predator and toxic agents (Anwar et al., 1984). This protective nature of bacterial biofilms was exploited for the development of an effective oral vaccine for finfish that can resist gastric destruction of epitopes, facilitating improved antigen delivery (Azad et al., 1999). The oral vaccination with biofilm cells of *Aeromonas hydrophila*, a common fish pathogen, elicited a significantly higher immune response and

In an experiment to enhance growth of common carp, rohu and tilapia through the use of sugarcane bagasse as substrate, Umesh et al. (1999) observed higher production of fish when bagasse was supplemented with cattle dung. This higher production of fish was attributed to bacterial biofilm promoted on the substrate which, apart from forming food for zooplankton and fish, contributed to improved water quality by lowering ammonia.

In another experiment, Joice et al. (2002) evaluated bacterial biofilm promoted on sugarcane bagasse in nursery for its effect on growth, survival and resistance to *Aeromonas hydrophila*, in hatchlings of common carp, *Cyprinus carpio*. It was observed that common carp grew faster under sugarcane bagasse treatment. The authors reported that fry reared in biofilm enhanced system had higher serum agglutination titre and protection against *Aeromonas hydrophila* compared to those from control and thus indicated the scope for improving the resistance of fish against ubiquitous secondary pathogens through biofilm production.

In a study to analyse the total protein, S-layer protein and LPS of biofilm cells of *A. hydrophila* by SDS-PAGE and to compare with that of planktonic cells, Asha et al. (2004) reported absence of S-layer protein and presence of an additional higher molecular weight band of LPS in biofilm cells compared to that in planktonic cells. The authors indicated that changes in the LPS profile might have contributed to the loss of S-layer. They also suggested that the high molecular weight band of LPS might play a role in the better performance of biofilm oral vaccine by eliciting a protective immune response.
Published works on the effect of bacterial biofilm on growth and immune response in shrimp culture system are scarce. Thompson *et al.* (2002) conducted studies to test the usefulness of biofilms in reducing the levels of ammonia and phosphate of rearing system water, and as food source for the shrimp *Farfantepenaeus paulensis*. The biofilm mass consisted of diatoms (*Amphore, Campylopyxis, Navicula, Sinedra, Hantschia and Cylindrotheca*) and filamentous cyanobacteria (*Oscillatoria* and *Spirulina*). The authors reported that pinnate diatoms and filamentous cyanobacteria were responsible for the largest uptake of ammonia from the water. It was suggested that the presence of biofilm lead to reduced exportation of phosphorous and to a higher output of nitrate + nitrite, instead of ammonia.