EVALUATION OF IMMUNO- BIOCHEMICAL ATTRIBUTES OF ASIAN GREEN LIPPED MUSSEL (*Perna viridis*) AND CHARACTERIZATION OF SELECTED IMMUNE RELATED GENE

By

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THESIS

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DECLARATION

I, hereby declare that the thesis entitled "Evaluation of immuno- biochemical attributes of Asian green lipped mussel (*Perna viridis*) and characterization of selected immune related gene" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Certified that this thesis entitled **"Evaluation of immuno- biochemical attributes of Asian green lipped mussel (*Perna viridis*) and characterization of selected immune related gene"** is a record of research work done by **Ms. Neethu B. Raj** (2014-09-120) under my guidance and supervision and that this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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DEDICATED TO MY BELOVED FAMILY

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LIST OF ABBREVATIONS

A260	Absorbance at 260 nm wavelength
A280	Absorbance at 280 nm wavelength
Abs	Absorbance
BCG	Bromo Cresol green
BHIA	Brain heart infusion Agar
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
cDNA	complementary DNA
CFU	Colony Forming Unit
dL	deciliter
°C	Degree Celsius
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy-nucleotide triphosphates
EDTA	Ethylenediamine tetra-acetic acid
Fig.	Figure
Frd	Forward
GPx	Glutathione Peroxidase

h	hour
H2O2	Hydrogen peroxide
H2S	Hydrogen sulphide
IPTG	Isopropyl β-D-thiogalactopyranoside
kDa	kiloDalton
LB	Luria Bertani
L-DOPA	L-dihydroxyphenylalanine
M	molar
MHA	Muller Hinton Agar
µg	Microgram
µl	Micro liter
min	minutes
ml	milliliter
NBF	Neutral Buffered Formalin
NBT	Nitro Blue Tetrazolium
NFW	Nuclease Free Water
nm	nanometre
O D	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
K2Cr2O7	Potassium dichromate
KOH	Potassium hydroxide

Pfu	*Pyrococcus furious*
pH	Log hydrogen ion concentration
RACE-PCR	Rapid Amplification of cDNA Ends
RNA	Ribonucleic acid
Rpm	Rotations per minute
Sec	second
NaCl	Sodium chloride
NaOH	Sodium hydroxide
SOD	Superoxide Dismutase
spp.	Species (plural)
Taq	*Thermus aquaticus*
TCA	Tri Chloroacetic Acid
TCBS	Thiosulphate Citrate Bile Salt Agar
THC	Total Hemocyte Count
UV	UltraViolet
X Gal	5-bromo-4-chloro-3-indolyl-β-D galactopyranoside
ZMA	Zobell Marine Agar

INTRODUCTION

1. INTRODUCTION

Perna viridis (Linnaeus) (Synonym: Asian green mussel) is one of the economically significant marine bivalves found all along the coastal areas of India (Rao, 1974). The high protein content and well-proportioned nutritional constituents of *P. viridis* make them a potential food source for human (Saritha and Patterson, 2015). The mussel is recognized as a well suited species of marine bivalve for culturing on a commercial scale due to its high population density and fast growth (Hickman, 1992; Rivonker *et al.,* 1993). Thus, increasing consumer demand and suitable characteristics for successful farming has resulted in a significant raise of mariculture practices of this bivalve in several locations within Kerala State and in different maritime States of India (Rivonker *et al.* 1993; Sreenivasan *et al.* 1996; Natarajan *et al.* 1997; Mohamed *et al.* 1998; Rajagopal *et al.* 1998; Sasikumar *et al.* 2000), but predominantly in northern districts of Kerala (Kripa and Mohamed, 2008).

Apart from the economic significance as food, *P. viridis* has also been recognized as an ideal aquatic animal for bio-monitoring (Nicholson, 2003). The filter feeding behavior of this animal can lead to higher accumulation of various pollutants in their tissues than the surrounding water. Therefore, use of different soft tissues of *P. viridis* for bio-monitoring of various pollutant's accumulation especially heavy metals and pesticides has been done by various authors (Chan, 1988; Krishnakumar *et al.* 1990; Nicholson, 2001, 2003; Siu *et al.* 2004; Nicholson and Lam 2005; Wang *et al.,* 2005; Yap *et al.,* 2006; Kamaruzzaman *et al.,* 2011). However, use of haemolymph and its immuno-biochemical attributes for monitoring of marine pollutants is rarely attempted.

The increasing interest in the culturing practices of *P. viridis* and application as bio-indicators for environmental monitoring necessitate the need for the basic data on various immunological and biochemical attributes of *P. viridis* in its natural

ecosystem. Therefore, the changes in immunological as well as biochemical parameters of haemolymph from these basal values can be applied in sustainable mariculture practices to predict various stressful conditions (like pathogen attack, variation in temperature, salinity and oxygen concentration) as well as in the environmental monitoring studies of various pollutants. Even though seasonal influences on the levels of antioxidant enzymes in various tissues of mussels have been correlated (Viarengo *et al.*, 1991; Wilhelm-Filho *et al.,* 2001; Manduzio *et al.,* 2004; Borkovic *et al.,* 2005), the corresponding data on immuno-biochemical parameters of haemolymph and mantle fluid is scarce.

On the other hand, studies regarding the characterization of immune genes coding for the different functions in *P. viridis* is not carried out. Ferritin is one of the significant immune related and critical multimeric proteins with major functions in iron storage and innate immunity in almost all living organisms. Ferritin is responsible for regulating levels of intracellular iron and has an essential role in iron withholding approach of innate immunity and detoxification mechanism in both vertebrates and invertebrates (Ong *et al.*, 2006; Li *et al.,* 2008). It has also been reported to be involved in the innate defense mechanism against pathogens infecting cultivated species of bivalves (Simonsen *et al.,* 2011; Zhang *et al.,* 2013; He *et al.,* 2013). Zhang *et al.* (2013) observed two H ferritin subunits in *V. philippinarum* (VpFer1 and VpFer2) and found out that the expression of this *ferritin* gene was increased during pathogen and heavy metal challenges. A novel ferritin subunit has been identified in pearl oyster *Pinctada fuctada* (Zhang *et al.,* 2003) and in *Chlamys farreri* (Chen *et al.,* 2016). However, ferritin have never been reported in *P. viridis*, and moreover, the gene is not yet fully characterized in any of mussels.

Thus the results of the study will serve as a reference for further investigation on the disease management studies on *P. viridis* as well as on the marine pollution monitoring studies using the same mussel.

Keeping in view of the above facts, the objectives of the present study was kept as follows:

- To analyze the seasonal impacts on biochemical and immunological parameters in the haemolymph and mantle fluid of *Perna viridis* and to characterize an immune related gene (*ferritin*) in *Perna viridis.*

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Perna viridis (Asian green mussel), is a valuable food source for human consumption and it is one of the candidate species for aquaculture. The review of literature regarding the research topic is included in this chapter.

2.1. *Perna viridis* (LINNAEUS, 1758) (SYNONYM: ASIAN GREEN LIPPED MUSSEL)

Perna viridis is one of the commercially and economically significant marine mussels under the family Mytilidae. It is also known as Philippine mussel, widely cultivated for profitable purposes especially in the Southeast Asian region. This species is extensively cultured due to its high productivity, high protein- vitamin content, high tolerance to a wide range of environmental conditions, and less farm management requirements (Rajagopal *et al.*, 2006; Al-Barwani *et al.*, 2007; McFarland *et al.*, 2013; Saritha and Patterson, 2015). Indo-Pacific region is the native habitat of Asian green mussel which includes areas between Japan to New Guinea, and from Persian Gulf to South Pacific Islands (FAO, 2013). *Mytilus edulis* L., a comparatively better studied blue mussel seen in higher latitude is the counterpart of this mussel (Bayne, 1976). *P. viridis* generally resides areas with high salinity and utilizes nutrients from land run-off (Rajagopal *et al.*, 2006). They are extensively seen in the east and west coast of India (Appukuttan and Mathew, 1987)

2.1. 1. Taxonomy:

Kingdom: Animalia
Phylum: Mollusca
Class: Bivalvia
Order: Mytilida
Family: Mytilidae

The genus *Perna* comprises four species, *viz*., the brown mussel *Perna* (Linnaeus, 1758), the green mussel *Perna viridis* (Linnaeus, 1758), the green-lipped mussel *Perna canaliculus* (Gmelin, 1791) and the Spanish mussel (Mediterranean green mussel) *Perna picta* (Born, 1780). New Zealand and Africa are the native habitat of *P. canaliculus* and *P. perna* (NIMPIS, 2002).

2.1. 2. Morphology and reproduction:

The normal size of *P. viridis* is 80-100 mm long and may reach up to 165 mm. The younger ones are bright green, and the color darkens as it matures. It has horizontally packed body encased in a shell which finishes in a descending pointing beak called umbo. Two pivoted shells are present, which are associated with a posterior adductor muscle (Gosling, 2015). One is a prolong shell, which is oval fit as a fiddle with swollen and pointed front and compacted posterior closures, a light blue sheen is present inside the shell. This mussel connects itself to the substrate by means of byssus threads (Seed and Richardson, 1999). External surface is smooth, yet a few sections have concentric development marks and faded outspread lines (Carpenter and Niem, 1998). The Asian green mussel has separate sexes and its fertilization happens externally. Color of reproductive organs is different for separate sexes; male have white gonads while female have bright red. A few studies demonstrated that temperature has some effect on its sexual development. (Lee *et al*., 1988). During spawning, the eggs and sperms are discharged into the water where one sperm fertilizes with single egg forming a circular shaped zygote. Transformation of the zygote to a larval stage takes 7-8 hours. This larva remains in water for 10-12 days and undergoes metamorphosis and accomplishes juvenile stage. At the juvenile phase it settles onto a surface, develops and attains sexual maturity in 2-3 months (Linnaeus *et al*., 1758) and finally achieves adult stage.

2.1.3. Behavior

Like every other mussel, green lipped mussels are sessile filter feeders principally feeds on phytoplankton, zooplankton and suspended organic materials (Defossez and Hawkins 1997; Burkhardt and Calci 2000). It flourishes in areas of

saltiness extending from around 18-33 ppt and temperature from 10-42°C. They produce byssus threads, an attachment fiber for mooring themselves to rocky surfaces, bridges, ships and other surfaces (McGuire and Stevely, 2015). As a defensive mechanism against predators like crabs and whelks, mussels increase their shell thickness and rises the byssus thread production for tightly anchoring itself to a surface, preventing these predators from separating or removing them from the attached substrate (Wong and Cheung, 2003). Asian green lipped mussel is highly sensitive to touch and temperature, and tightly close their shell when it is disturbed. Sensors are present in them for identifying the chemical changes in marine environment (Wong and Cheung, 2003).

2.1.4. Significance in terms of human health perspectives:

Asian green lipped mussel is presently considered as a cheap protein source, containing high dietary benefits and it is famous for its delicious taste (Rajagopal *et al.*, 1998). Total protein, carbohydrate, lipid, amino acids, vitamins and mineral contents are the major biochemical constituents present in these mussels (Devadason, 2015). The major nutrient is protein followed by carbohydrate and lipid. Amount of total essential amino acids is higher than the non-essentials. Seven vitamins were detected, among which vitamin A and C are predominant ones. Six macro minerals and 2 trace minerals were also identified in this mussel (Saritha and Peterson, 2015). Thus, this mussel is presently considered as a highly nutritious human food with a variety of vitamins, minerals, amino acids, Omega-3 fats and antioxidants (Chakraborty, 2011). Gopalakrishan and Vijayavel (2009) showed that *P. viridis* exhibits a good nutritional composition compared to two other mussels namely, *Donax cuneatus* and *Meretrix meretrix* in terms of its biochemical constituents (protein, carbohydrate, lipid and vitamins and minerals including iron, copper, zinc, calcium, magnesium, and manganese. The calorific value of the meat was as 6.28 Cal/gm (dry weight) (Kuriakose and Appukuttan, 1980).

Apart from the nutritional significance, the extracts prepared from this mussel were found to have therapeutic values in terms of chondro-modulation as well as anti-

inflammatory properties (Sreejamole *et al.*, 2011; Chakraborty *et al.*,2019). Sreejamole and Radhakrishnan (2013) indicated that ethyl acetate extract of *P. viridis* has cytotoxic and antioxidant properties which can be utilized in cancer treatment strategies and to fight against oxidative stress-induced diseases. The importance of *P. viridis* as a source of anti-HIV activity was demonstrated by Mitra *et al.* (1996).

2.1.5. Applications of *P. viridis*

Perna viridis has become one of the economically significant marine bivalves due to its high nutritional value and various therapeutic applications. The mussel species is now recognized as a well suited species of marine bivalve for culturing on a commercial scale due to its high population density and fast growth rate (Hickman, 1992; Rivonker *et al.*, 1993). Thus, increasing consumer demand and suitable characteristics for successful farming has resulted in a significant raise of mariculture practices of this bivalve in several locations within Kerala State and in different maritime States of India (Rivonker *et al.* 1993; Sreenivasan *et al.* 1996; Natarajan *et al.* 1997; Mohamed *et al.* 1998; Rajagopal *et al.* 1998; Sasikumar *et al.* 2000), but predominantly in northern districts of Kerala (Kripa and Mohamed, 2008).

Apart from the economic significance as mentioned above, *P. viridis* has also been recognized as an ideal aquatic animal for bio-monitoring (Nicholson, 2003). The filter feeding behavior of this animal can lead to higher accumulation of various pollutants in their tissues than the surrounding water. Therefore, use of different soft tissues of *P. viridis* for bio-monitoring of various pollutant's accumulation especially heavy metals and pesticides has been done by various authors (Chan 1988; Krishnakumar *et al.* 1990; Nicholson 2001, 2003; Siu *et al.* 2004; Nicholson and Lam 2005; Wang *et al.*, 2005; Yap *et al.*, 2006; Kamaruzzaman *et al.*, 2011).

2. 2. MOLLUSCAN MARICULTURE

Mariculture is the rearing and farming of marine organisms and plants for food and different products like drugs, food additives, jewelry (pearls),

nutraceuticals, and cosmetics under controlled or semi conditions in briny water or marine environment. From ancient times onwards mariculture is getting great importance globally as it provides potentials for sustainable food production and economic progress of local societies. Among Asian countries China is the major producer of mariculture products with nearly 22 million tonnes of farmed marine species. Other Asian countries involved in mariculture include Indonesia, Malaysia, Philippines, Singapore, South Korea, Srilanka, India, Taiwan and Thailand (FAO, 2004).

Molluscan mariculture constitutes about 18% of the total world mariculture production. Among various molluscs, mussels (31%) followed by oysters (27.2%), clams (21.9%) and scallops (15.7%) are the major candidates for mariculture. Bivalve cultivation is a self-manageable aquaculture that requires no extra feed and minimal maintenance work (Tan and Ransangan, 2014) and is considered to have less environmental impact compared to finfish culture. Along with these benefits, bivalves have the ability to uphold, modify and build entire habitats due to their impacts on suspended particles and their shell formations. Due to this reasons bivalve mariculture is getting significance among the farmers. The success of bivalve culturing is greatly reliant on water quality and a healthy ecosystem (Gallardi, 2014) and its management encompasses the thinning of the bivalves where culture density is too high to support optimal growth and development, checking for and controlling predators and biofouling (Philips, 2009). Some high-value mollusc species like abalone are cultured in land-based tanks, but most mollusk farming occurs in the sea, where three actual frameworks are mostly utilized: Within-particulate substrates – this framework is utilized to culture substrate-occupying cockles, mollusks etc. Second is 'On or just above the bottom'-this culture framework is often utilized for culture of bivalves that endure intertidal exposure, for example, clams and mussels. Third is surface or suspended culture- cultivation of bivalves on ropes or in containers, suspended from floating rafts (Mohan Joseph 1998; Lucas, 2003).

2.2.1. Molluscan mariculture in India

The culturing of marine molluscs such as pearl oysters, clams, mussels and their hatchery methods for seed production have been developed in India during 70s, mainly due to the efforts at the Central Marine Fisheries Research Institute (CMFRI) (Appukuttan *et al.,* 1996). Even though the culturing methods have proven to be technologically viable it was not greatly adopted by farmers at that time due to the lack of knowledge and finance. In 1993, Government of India introduced various action programmes for disseminating the farming technology for molluscs along the selected areas of southwest India and got remarkable results. The indigenously developed method for the production and culturing of Indian pearl oyster (*Pinctada flucata*) has been considered as the major technological accomplishment in Indian mollusc mariculture and this technology attained immense attention among the farmers (Appukuttan *et al.,* 1996). The major producers of marine molluscs in India include Kerala, Andhra Pradesh, west Bengal, Tuticorn and Andaman (Laxmilatha, 1996). The main candidate species targeted for molluscan mariculture practices of India were depicted in Table 2. 1.

Table 2. 1 Candidate species of molluscan mariculture in India

Groups	Scientific name	Common name	Reference
Mussels	*Perna viridis* *P. indica*	Asian green lipped mussel Indian brown mussel	Silas, 1980
Oysters	*Crassostrea madrasensis* *C. gryphoides* *C. rivaluris*	Indian backwater oyster Giant oyster Chinese oyster	Muthiah, 1998
Clams	*Metetrix casta* *M. meretrix* *Paphia malabarica,* *Anadara granosa* *Villorita cyprinoides*	Backwater clam Asiatic hard clam, great clam Short neck clam Blood clam Black clam	Narasimham, 1998
Pearl oyster	*Pinctada fucata* *P. margaritifera*	Akoya pearl oyster Black lip pearl oyster	Chellam, 1991

2.3. *P. viridis* for bio-monitoring

As *P. viridis* are filter-feeders, they concentrate and accumulate various insoluble contaminants in their tissues in a concentration which is considerably above that present in their surrounding water (Connor, 1999). This bioaccumulation property in different tissues have been exploited for bio-monitoring of various pollutants in Asia Pacific coastal areas (Tanabe *et al* 2000; Nicholson and Lam 2005) including India (Chidambaram, 1991), China (Fung *et al.*, 2004), Malaysia (Yap *et al.*, 2003; Yap *et al.*, 2004). Thus, Asian green lipped mussel has been extensively used in mussel watch programs and as a pollution indicator throughout Indo-pacific areas to assess the toxic effects of a broad range of contaminants including heavy metals, organochlorines, PAHs *etc.* (Nicholson, 2003).

Various changes in this organisms' body compared to a control site has been considered as biomarkers of environmental pollutants which comprises molecular, physiological, cytological and histological biomarkers (Nicholson and Lam, 2005). Many immune parameters in different tissues of *P. viridis* have also been identified as potential which include antioxidant enzyme system, phenol oxidase activity, lysosomal membrane stability and phagocytosis biomarkers (Nicholson and Lam2005). Many environmental factors induce the production of reactive oxygen species (ROS) and activation of several antioxidant enzymes for protecting the cells from the ROS toxicity. Therefore, appropriateness of various antioxidant parameters, such as glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione (GSH) and lipid peroxidation, for application as biomarkers has been observed in *P. viridis* (Cheung *et al.*, 2001; Lau and Wong, 2003). Jena *et al.* (2012) found out that the cellular damage and oxidative stress indices in the digestive gland tissue of *P. viridis* could suitably be used as biomarkers of petroleum pollution. Physiological indices such as heart rate, clearance rate, condition index, growth rate, shell growth and byssus production are also widely recognised as the appropriate and suitable biomarkers in these mussels (Nicholson, 2003; Nicholson and Lam, 2005). However, use of haemolymph and its immuno-biochemical attributes for monitoring of marine pollutants is rarely attempted in *P. viridis*.

2.4. IMMUNOLOGY OF MOLLUSCS

2.4.1. Immune system

Molluscs depend solely on an innate, non-lymphoid system of immune responses mediated by both cellular and humoral components (Loker *et al.,* 2004). The cellular components comprise phagocytosis or encapsulation, with pathogen destruction by enzymes and release of oxygen metabolite while, humoral components include several reactions mediated by sequence of molecules (Wootton *et al.,* 2003).

Molluscs have an open circulatory system with haemolymph as their circulating fluid which is always exposed to environmental factors and pollutants. The haemolymph contains cells (known as hemocytes) which represents the most significant internal defense mechanism. Invertebrate hemocytes are responsible for various physiological processes like recognition, phagocytosis, and elimination of non-self-particles (Pipe, 1992; Chu, 2000; Canesi *et al.,* 2002). It also has significant part in gas exchange, osmoregulation, digestion, transport, excretion, repairing of tissues and internal defense (Cheng, 1981; Lopez *et al.,* 1997). The role of hemocytes in invertebrates is similar to monocyte and macrophages in the vertebrates. Non-self-particles mainly activates cell mediated immune responses in bivalves, which mainly include proliferation of hemocytes, engulfing of non-self-particles, production of toxic reactive oxygen species (ROS), respiratory burst which involves the production of oxygen metabolites, such as superoxide anion, hydrogen peroxide and variations in anti-oxidative enzymes (Lopez *et al*., 1997; Roch, 1999) In mussels haemolymph also functions as a fluid frame, providing firmness and strength to some organs such as the labial palps, foot, and mantle edges. Along with these functions hemocytes acts as a key element for the transportation of contaminants from the gills and mantle to the tissues where it accumulates (Ruddel and Rains 1975; George *et al.,* 1978). The two major types of hemocytes observed in mussels are granulocytes and hyalinocytes (Cajaraville and Pal, 1995). Granulocytes are the major haemocytes (~90% of the total). They have larger granulocytes with higher phagocytic activity, while hyalinocytes are usually smaller than granulocytes, and have a high nucleus/cytoplasm ratio (Hine *et al.,* 1999; Matozzo *et al.,* 2007). The effect of physiological disturbances in mussels can be seen primarily in hemocytes, so that toxic and adverse effects of different chemical contaminants to the organism can be assessed by monitoring several immunological and biochemical parameters of the mussel immune system (Wong *et al.,* 1992). The variations in haemolymph cellular composition, total and differential counts and significant cell mortality are considered as the biomarkers for environmental stress in almost all the bivalves (Anderson,

1988; Fisher, 1988). It has been observed that bivalve haemocyte counts may be reduced by a high level of selected heavy metals (Fries and Tripp, 1980; Cheng and Sullivan, 1984).

2.4.2. Immuno-biochemical attributes

Various immunological attributes of haemolymph in molluscs include lysozyme, acid phosphatases, esterase, β-glucuronidase, phenoloxidase, ROS (reactive oxygen species) production and activity of various antioxidant enzymes. Biochemical attributes mainly include the level of glucose, glycogen and protein. The significance of each attribute can be summarized as follows.

Among all the immune parameters, ROS production and activity of various antioxidant enzymes are considered as the most significant ones in molluscs (Winston and Di Giulio, 1991). Production of (ROS) is a major mechanism for cell disruption and killing. There is a balance maintained between ROS generation and neutralization under normal conditions. An increase in respiratory burst and production of ROS is observed when haemolymph is stimulated by any external element. This is due to the generation of H_2O_2 in response to xenobiotics due to the activation of an enzyme complex which catalyzes conversion of molecular oxygen. This ultimately leads to the production of many monovalent and many cytotoxic ROS like superoxide, hydrogen peroxides and hydroxyl radicals (Thannickal and Fanburg, 2000). Production of superoxide radicals and other toxic metabolites that destroys the invading pathogens has been demonstrated in many bivalves such as *M. edulis* (Pipe, 1992), *M. galloprovincialis* (Pipe *et al.*, 1995; Carballal *et al.,* 1997), *P. viridis* (Adema *et al.*, 1991) and the oyster *C. virginica* (Fisher *et al.,* 1996). This cellular defense reaction is immunologically important and has been well identified in almost all molluscs (Adema *et al.* 1991; Arumugam *et al.* 2000). Changes in ROS production has been reported in the mussels *M. galloprovincialis* (Carballal *et al.,* 1997; Pipe *et al.,* 1995), *M. edulis* (Winston *et al.*, 1996), *P. viridis* (Thiagarajan *et al.,* 2006) and oyster *C. virginica* (Anderson, 1994) after exposure to xenobiotics. Antioxidant enzymes are those which protect the host cells from the oxidative stress damage

produced by this ROS accumulation. Various components of the detoxification and antioxidant system in molluscs have been shown to be specifically induced by metals and chemical contaminants (Regoli and Principato, 1995). Various laboratory and field studies have been showed that changes in the levels or activities of antioxidants are potential biomarkers for pollution monitoring (Porte *et al.*, 1991; Ribera *et al.*, 1991; Verlecar *et al.*, 2008). Superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GSH-Px, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2) and Glutathione-S-transferase (GST, EC 2.5.1.18) are the major antioxidant enzymes involving in reduction of ROS levels and are mainly considered as biomarkers of chemical pollutants (Nicholson and lam, 2005). Among these antioxidants, superoxide dismutase is the primary and the most vital defense line. It is a free radical elimination enzyme which can minimize the oxidative damage of host cells produced by external stress (Zhang *et al.*, 2005). It catalyzes the dismutation of superoxide radical, the common by-product of oxygen metabolism (Hassan *et al.*, 1988). Another commonly studied antioxidant enzyme is catalase which has an important role in primary anti-oxidative mechanism (Cajaraville *et al.*, 2000; Khessiba *et al.*, 2001). Glutathione peroxidase is a peroxidase enzyme that catalyzes the conversion of lipid hydro peroxides and hydrogen peroxides to corresponding alcohols and water respectively (Winston and Di-Giulio, 1991). Application of these antioxidant enzymes as biomarkers has been examined in various tissues of *P. viridis* by Cheung *et al.* (2001) and Lau and Wong (2003). Reduced Glutathione (GSH) is considered as another main cofactor of enzymes catalyzing the detoxification of several poisonous compounds entering the organism's body and also functions in maintaining the normal metabolism of mussels (Meister, 1989).

Another main immunological response to pathogenic attack in molluscs is the activation and action of lysosomal enzymes which help in destruction of pathogen by hydrolytic action (Cheng, 1978). Lysozyme, acid phosphatases, esterase and β-

glucuronidase are the main enzymes involved in this process (Sun and Li. (1999), Mazorra, *et al.,* 2002). Aminopeptidase (Yoshino and Cheng, 1976; Oubella *et al.,* 1994), lipase (Pipe, 1990), serine protease (Bachere *et al.*, 1990), and phenoloxidase (Asokan *et al.*, 1997; Carballal *et al.,* 1997) are the other enzymes that get activated at the time of stress.

Melanisation, the process involving in capsule formation and tissue repairing in arthropods is considered as part of immune system in bivalves (Coles and Pipe, 1994; Asokan *et al.*, 1997; Aladaileh *et al.*, 2007). Phenoloxidase (PO) is the key enzyme in this complex enzymatic reaction that leads to the generation of melanin as the last compound (Soderhall and Cerenius, 1992; Ashida and Brey, 1997). The enzyme PO exists in the haemolymph in an inactive proform (proPO) which is activated through a proteolytic cleavage by serine proteases. This serine protease can be activated by cell wall components of microbes, such as LPS and peptidoglycans from bacteria and α-1, 3-glucans from fungi (Soderhall and Cerenius, 1992; Ashida and Brey, 1997). In crustaceans and molluscs, PO activity appears more in hemocyte vesicles than plasma (Soderhall and Cerenius, 1992). However, a few reports are available on the presence of PO activity in mussels. Asokan *et al.* (1997) reported the PO activity in the plasma and haemocyte fraction of the mussel *P. viridis* after induction by trypsin, LPS and α-1, 3-glucans.

Variations in major biochemical elements in the haemolymph like glucose, glycogen and protein are also applied as indicators of the disruption of the oxidative carbohydrate metabolism and stress indicators in molluscs (Lagadic *et al.*, 1994). Carbohydrates are considered as the first organic depleted when the animal is under any kind of stress (Clark, 1975). Cholesterol has been reported as the major and predominant sterol present in most of the bivalves (Murphy *et al.*, 2002).

As mussels are filter feeding organisms they generally exposed to a huge number of various microorganisms including Achromobacter spp., Aeromonas spp., Alcaligenes spp., Fiavobacteium spp., Pseudomonas spp. and Vibrio spp. organisms

and Bacillus spp (Olafsen *et al.*, 1993) and accumulates these microrganisms in their body. Marine molluscs are considered as the potential source of antimicrobial products. Different extracts of *Perna viridis* has showed both antibacterial and antifungal activities. (Kiran *et al.*, 2014; Sumita *et al.*, 2009). There are reports about the antimicrobial, antioxidant, anticancer and antiulcer properties of *P. viridis* extracts (Annamalai *et al.*, 2007; Kiran *et al.*, 2014; Madhu *et al.*, 2014: Venketaswaran *et al.*, 2017).

The various immunobiochemical attributes and their normal ranges reported in the haemolymph of the targeted organism of the present study (*P. viridis*) are given in Table 2.2.

Table 2.2 Immuno-biochemical attributes in the haemolymph of *P. viridis*

Immuno-biochemical parameters	Normal values	Reference
Total hemocyte count	$1.85\pm0.031*10^6$ cells/ml	Bindya *et al.* (2010)
Glucose	1.06 mg/ml	Bindya *et al.* (2010)
Protein	1.76 mg/ml	Bindya *et al.* (2010)
Cholesterol	Not studied	
Triacylglyceride	Not studied	
Phenoloxidase	0.008±0.003	Asokan *et al.* (1997)
Superoxide anion generation	0.063± 0.0014	Bindya *et al.* (2010)
Lysozyme	Not studied	

2.4.3. Immune related genes:

Many immune related genes have been identified in molluscs and these are found to be over-expressed in response to various environmental stress as well as pathogenic attack (Table 2.3). The major genes include genes encoding various Pathogen Recognition Receptors (PRRs) including Toll-like receptors, genes encoding antimicrobial peptides (AMPs), genes encoding C type lectins and various cytokines, details of which are given below.

Toll-like receptors (TLRs) are one type of PRRs which are linked with recognizing pathogens invading the cells and in activating the immune system against the pathogens, particularly in innate immune defense mechanism of the host. Toll like receptors and its significance in immune responses have been identified in most of the molluscs (Song *et al.*, 2007; Toubiana *et al.,* 2013). C-terminal fibrinogen-like domain (FREPs) are another important and best studied families of Pathogen Recognition Receptors in molluscs (Hanington *et al.*, 2011; Adema *et al.*, 2015).

Antimicrobial peptides (AMPs) are recognized as the key mediators of the first line defense mechanism against microbial attack. Various anti-microbial peptides have been reported in molluscs including defensins, big defensins, mytilins, myticins, mytimycins *etc* (Mitta *et al.,* 1999; Gueguen *et al.,* 2006; Venier *et al.,* 2011; Rosani *et al.,* 2014).

Another immune related gene is C-type lectins which has significant role in innate immune recognition (Zelensky and Gready, 2005). The role of C-type lectins in the molluscan immune system has been clearly established and thoroughly investigated (Wang *et al.,* 2011). Molluscan C-type lectins are reported to agglutinate bacterial cells and are up-regulated by bacterial challenges in *C. farreri* and *C. virginica* (Jing *et al.,* 2011; Zhang *et al.,* 2015). Cytokines such as TNFs and interleukin 17 (IL-17) and their receptors are also reported to plays a key role as regulators of immunity, inflammation and apoptosis. In *C. gigas* many cytokines such as TNF, TRAF etc. were identified and found up-regulated by bacterial challenges (He *et al.*, 2015; Zhang *et al.*, 2015).

Table 2.3 Immune related genes identified in molluscs

Immune related genes/ Protein	Mollusc	Reference
Defensin	*Haliotis discus, Hyriopsis schlegelii, Crassostrea gigas, Ruditapes philippinarum, Venerupis philippinarum*	Whang *et al.* (2010) ; Zhao *et al.* (2010); Rosa *et al.* (2011); Peng *et al.* (2012) ; Wang *et al.* (2015)
C1q gene family	*Crassostrea gigas, Chlamys farreri*	Zhang *et al.* (2008) ; Gerdol *et al.* (2015)
R type lectin	*Mytilus galloprovincialis*	Hasan *et al.* (2016)
TRAF 6	*Mizuhopecten yessoensis*	Wang *et al.* (2013)
nfkB	*Patinopecten yessoensis*	Zhang *et al.* (2015)
C type lectin	*Crassostrea virginica.*	Jing *et al.* (2011)
Rel\NF-κB	*Haliotis diversicolor*	Jiang *et al.* (2007)
MyD88	*Patinopecten yessoensis*	Ning *et al.* (2015)
Mytimycin	*Mytilus galloprovincialis*	Sonthi *et al.* (2011)
Toll-like receptors	*Mytilus sp.*	Toubiana *et al.* (2013)
Myticin-C	*Mytilus galloprovincialis*	Vera *et al.* (2011)
IkappaB	*Pinctada fuctada*	Zhang *et al.* (2009)
Fibrinogen-related proteins	*Crassostrea gigas*	Zhang *et al.* (2012)

2.4.3.1. Ferritin and its role in innate immune response of molluscs

Ferritin is an important protein found in almost all living organisms, primarily functions in iron storage and in regulation of intracellular iron levels (Gaymard *et al.*, 1996; Petit *et al.*, 2001; Vanarsa *et al.*, 2012). It has been identified as a significant protein in maintaining iron homeostasis in both vertebrates and invertebrates

(Gaymard *et al.,* 1996; Zhang, 2011). This iron-binding protein also prevent iron toxicity and toxic hydroxyl radicals produced during various redox reactions (Hamburger *et al.,* 2005; Vanarsa *et al.,* 2012; Theil, 2013). It is highly stable to temperature and extreme pH values. Ferritin protein was first identified from horse spleen by Laufberger (1937) and named by Granick and Michaelis (1942). Most organisms are found to contain many functional *ferritin* gene including bacteria. There may be more than three *ferritin* genes in some animals while some has just one *ferritin* gene, (Zhu *et al.,* 2012). Ferritin is composed of 24 subunits that assemble and form a dodecahedron of 12 nm in diameter, with a large hollow cavity 8 nm across (Lawson *et al.,* 1991). Natural ferritins are purified as iron-containing proteins and the iron is readily extracted by reducing agents. Insect ferritin is mainly secretory and consisted of two subunits H- and L type (Gutierrez *et al.,* 2013). It has been reported that this protein has involvement in the innate defense against viruses and pathogens infecting cultivated species of insects (Simonsen *et al.,* 2011). The oyster *Crassotrea gigas* has been reported to have four distinct *ferritin* genes (two for cytosolic and two for secretory ferritins) (Huan *et al.,* 2014). All four have different expression patterns in the tissues and during developmental stages, signifying some functional differences (Yao *et al.,* 2015). Zhang *et al.* (2013) showed that many shrimps express single ferritin type which is alike to H-ferritin and these are get overexpressed by some immunochallenges and by viral infection (Feng *et al.*, 2014). A novel ferritin subunit showing similarity with H subunit type of mammalian ferritins has been identified in pearl oyster *Pinctada fuctada.*

2.5. VARIATIONS IN IMMUNO-BIOCHEMICAL PARAMETERS OF MOLLUSCS

Immune defensive capability and biochemical attributes of molluscs change in accordance in response to biotic and abiotic stresses. Abiotic stresses including environmental variations like change in temperature, dissolved oxygen and salinity have been shown to have immense effect on various immuno-biochemical parameters (Gagnaire *et al.,* 2006; Ottaviani *et al.,* 2007) and metabolism of the marine

organisms (Vernberg, 1972). Biotic stress like stress during captivity was found to have effect on the biochemical variables in haemolymph of shrimps (Sanchez *et al.*, 2001). The most important factors leading to the variations in immuno-biochemical parameters of bivalves are described below.

2.5.1. Temperature

The variations in temperature produce alterations in most of the defensive mechanisms and physiological processes of mollusks, finally causing the death of the animal (Terahara *et al.*, 2008). When water temperature reaches above or below a particular limit the normal oxygen metabolic rate of mussels decreases and change to anaerobic metabolism even if the water is abundantly oxygenated (Sokolova and Pörtner, 2001). The exposure to high temperatures mainly elevates the accumulation of ROS and antioxidant enzyme activities (Murphy, 2009). When the antioxidant system is collapsed, a drastic increment in ROS production happens and causes the lipid peroxidation of cellular membranes and tissue damage, finally results in the loss of major physiological processes (Manduzio *et al.,* 2005). A higher increase in ROS production and antioxidant enzymes were detected in tissues of *P. viridis and Mytilus sp.* after exposure to temperature stress (Verlecar *et al.,* 2008; Wu *et al.*, 2016; Wang *et al.*, 2018. Variations in temperature were reported to cause changes in hemocyte mediated immune responses such as phagocytosis, ROS production as well as activation of antioxidant enzymes like SOD, GPX and catalase etc. in molluscs such as American oyster (Fisher and Newell, 1986) *Ostrea edulis* and *Crassostrea virginica* (Fisher and Newell, 1986). Wang *et al.* (2018) showed that cold and heat stress prompted increment in hemocyte mortality and ROS production in *P. viridis.* However, antioxidant enzyme activities were found to be enhanced only in heat stress.

2.5.2. Dissolved oxygen

Hypoxia is a condition of low dissolved oxygen concentration in water which negatively affects the aquatic organisms causing behavioral, physiological, biochemical and molecular responses (Zhong *et al.,* 2009; Gui and Zhu, 2012).

Studies showed that hypoxia could change the immune parameters of bivalves, such as hemocyte count, hemocyte mortality, phagocytic activity, lysozyme activity, antioxidase activity and ROS production (Matozzo *et al.,* 2005; Yu *et al.,* 2010) Exposure to environmental hypoxia lessens the capability of mollusc haemocytes to produce ROS (Boyd and Burnett, 1999, Cheng *et al.,* 2004). Pampanin *et al.* (2002) reported that air exposure negatively affected hemocyte functionality of *Chamelea gallina,* resulting in a decrease in hemocyte count, binding capacity, phagocytic efficiency and enzyme activity. It has been demonstrated that *Chamelea gallina* (Matozzo *et al.*, 2005; Monari *et al., 2005)* and *Mactra veneriformis* (Yu *et al.,* 2010) produced various immunological changes in response to anoxia condition.

2.5.3. Salinity

Salinity is a key environmental factor which has importance in health and growth of marine organisms. Changes in salinity are known to influence metabolic and physiological parameters in bivalves including heart rate (Bakhmet *et al.,* 2005), respiration (Stickle and Sabourin, 1979) and growth rate (Westerbom *et al.,* 2002). Reduced salinity caused a substantial reduction in total hemocyte count and phagocytic activity in mussel *Mytilus edulis* (Bussell *et al.,* 2008). A study by Gagnaire *et al.* (2006) reported that low salinity reduced the hemocyte mortality, esterase and phagocytosis in the Pacific oyster *Crassostrea gigas.* Some previous studies with *P. viridis* has revealed that exposure to sub-lethal salinities result in detrimental variations in behaviors such as closing of shells and weak attachment using threads (Farland *et al.,* 2015) and change in immunological parameters like high haemocyte mortality, low THC, decreased phagocytosis, Reactive oxygen species and lysosomal content (Wang *et al.,* 2012). Wang *et al.* (2012) reported that hypo salinity and hypoxia led to low ROS production inducing poor oxidative defense in *P. viridis.*

The factors which are reported to influence the immuno-biochemical attributes of *P. viridis* are in summarized in Table 2.4.

Table 2.4 Influencing factors of immuno-biochemical attributes in *P. viridis*

Influencing factor	Parameters influenced	Tissues /fluid	Change	Reference
Temperature	Superoxide anion generation	Gills	Increased	Wang *et al.* (2018)
	SOD	Gills	Increased	Wang *et al.* (2018)
	Glutathione peroxidase	Gills	Increased	Wang *et al.* (2018)
	Catalase	Gills	Increased	Wang *et al.* (2018)
	ROS	Gills	Increased	Wang *et al.* (2018)
Salinity	Total hemocyte count	Haemolymph	Decreased	Wang *et al.* (2012)
	Haemocyte mortality	Haemolymph	Increased	Wang *et al.* (2012)
	ROS	Haemolymph	Decreased in low salinity	Wang *et al.* (2012)
Dissolved oxygen	ROS	Haemolymph	Low during hypoxia	Wang *et al.* (2012)

MATERIALS AND METHODS

3. MATERIALS AND METHODS:

The present study was carried out at Marine Biotechnology Division, ICAR-CMFRI. The material and methods used for the study during the year 2018-2019 are elaborated in this chapter.

3.1 MATERIALS

3.1.1. EQUIPMENTS

Cooling micro centrifuge (Remi, India),Thermal cycler (ABI Veriti 96 well Thermocycler, Thermofischer scientific and Proflex, India), Digital pH meter (Elico Ltd, India), Vortex mixer (Spinix, India), Laminar flow system (Labline, India), UV trans illuminator (Gelstan, India), Orbital shaker cum incubator (Labine, India), Microwave oven (Samsung, India), Incubator (Kemi, India), Electronic weighing balance (Shimadzu, India), Hot air oven (Kemi, India), Autoclave (Labine, India) Refrigerator(Samsung, Whirlpool, India) Ultra low temperature freezer -80°C (New Brunswick Scientific, India), Microscope (Leica micro system, India), Spectrophotometer (Multiskan Go, Thermofischer Scientific), Bio photometer (Eppendorf), water bath (Labline, India), refractometer (Cole Parmer India Pvt Ltd) were used in the present study.

3.1.2. GLASS WARE AND PLASTIC WARE

Test tubes, petridishes, conical flasks, beakers, glass rods etc. used in this study were procured from reputed firms such as Borosil (India). They were thoroughly washed and sterilized as per standard procedures. Plastic wares including micro centrifuge tubes, micropipette tips and centrifuge tubes were sterilized and used.

3.1.3. CHEMICALS AND REAGENTS

Chemicals and reagents used have been mentioned either at appropriate places or listed in the Appendix. Chemicals were from reputed firms such as Sigma and HI media (India), and other reputed firms. The compositions of media are enclosed in Appendix or at appropriate places.

3.1.4. BACTERIAL CULTURES USED IN THE STUDY:

A total of six aquatic pathogens were used for the evaluation of antibacterial activity of haemolymph and mantle fluid. Details were given in the table 3.1.

Table 3.1. Bacterial strains used in the study

Pathogens	Strain ID	Source
Vibrio harveyi	CMFRI/VHa-03	Marine microbiology laboratory, ICAR-CMFRI, Kochi
Vibrio vulnificus	CMFRI/VV-01	Marine microbiology laboratory, ICAR-CMFRI, Kochi
Vibrio anguillarum	CMFRI/Van-01	Central Institute of Brackish water Aquaculture, Chennai
Vibrio parahaemolyticus	CMFRI/VP-08	Marine microbiology laboratory, ICAR-CMFRI, Kochi
Vibrio alginolyticus	CMFRI/ VA1-01	Central Institute of Brackish water Aquaculture, Chennai
Photobacterium damselae	CMFRI/PhD-05	Marine microbiology laboratory, ICAR-CMFRI, Kochi

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3.1.5. PRIMERS USED IN THE STUDY

Various primers used in the present study are shown in Table 3.2.

Table 3.2 Primers used in the study

Sl. No.	Primer name	Primer sequence (5'-3')	Reference
1	Fer D1	5'GCCWSCTACRBCTAYCWGTCIATG3'	Zhang *et al.*, 2003
2	Fer D2	5'CTCCTTRATIGCYTYCACYTGYTG3'	Zhang *et al.*, 2003
3	Fer 3 For	5'GAGGAGAGAAGAACATGCTGAGA3'	Present study
4	Fer 5 Rev	5' CAGCATGTTCTCTCTCCTCATCTG 3'	Present study
5	Fer 5'cDNA	5' CTCACGGTCATAGATGTACTCCC3'	Present study
6	dT anchor	5'GAC CAC GCG TAT CGA TGT CGA CTT TTT TTT TTT TTV3'	5'-3' RACE kit, Roche
7	PCR anchor	5'GACCACGCGTATCGATGTCGAC3'	5'-3' RACE kit, Roche
8	M13 for	5'GTAAAACGACGGCCAGT3'	Universal primer
9	M 13 rev	5'GGAAACAGCTATGACCATG3'	Universal primer

3.2. METHODS

3.2.1 SAMPLE COLLECTION AND MAINTENANCE

Perna viridis samples were collected from the Njarackal sea coast, Cochin, India during three different seasons namely cool dry season (November-February), humid summer season March-May) and Monsoon season (June-September) (Plate.3.1). A least polluted site away from industrial and urban interventions were chosen for sample collection. A total of 36 healthy mussels (pooled to 6 groups per each season) were randomly collected for each season from natural rocky beds in sub tidal regions using diving method. To reduce the handling stress, samples were kept in an ice box, transported to the laboratory immediately after collection and maintained at wet lab, ICAR- Central Marine Fisheries Research Institute for 48 hrs. in sea water (having the same salinity as that of the sampling site) in a fibre-glass tank (500 L) equipped with a filtering system and air supply. The experimental animals were fed with sea algae (*Nanochloropsis sp.*) once in 2 days in the facility. Prior to experiment, the morphometrics were recorded for each group of animals. (Table 3.3). The Shell length of animals was varied from 4.1 to 5.5 cm on cool dry season (November-February), 4.9 to 8.5 cm on humid summer season (March-May) and 5.5 to 9.3 cm on Monsoon season (June-September). Condition index of each individual were calculated by the method of Abbe and Albright (2003). Hydrographic parameters of the corresponding resident water were recorded during each sampling per season. Water salinity at the collection site was measured using a refractometer (Cole Parmer India Pvt Ltd). The pH of surface water was measured using a Digital pH meter (Elico Ltd, India).

3.2.1.1 Haemolymph collection and preservation

All the animals were blotted dry prior to haemolymph collection. About 1.5 ml haemolymph was collected aseptically from the posterior adductor muscle of each *P. viridis* using a 1 ml plastic syringe with a needle (0.75×38 mm) according to

Gustafron *et al.* (2005) and stored in ice. Special care was taken not to rupture the adductor muscle while collecting the haemolymph. Haemolymph of six animals was then pooled into tubes to reduce individual variation as well as to provide sufficient haemolymph for the assays carried out in the present study. The haemolymph samples were kept in 4°C until use.

3.2.1.2 Mantle fluid collection

Mantle fluid was collected carefully and aseptically using a 26 gauze hypodermic needle fitted to a 2 ml syringe from the mantle cavity. Care was taken to avoid the rupture of the mantle and leakage of fluid at the time of opening the mantle cavity. The fluid collected was kept at 4^0C.

Plate 3.1 *Perna viridis*

Table 3.3 Average morphometric data of each animal groups collected in three seasons

Seasons	Groups	Average weight, g	Average length, cm
Cool dry season	1	8.11± 0.516	4.69± 0.25
	2	8.208 ± 1.19	4.925 ± 0.34
	3	7.61± 2.23	4.608 ± 0.48
	4	8.10 ± 0.50	4.67± 0.25
	5	7.59± 2.21	4.61 ± 0.47
	6	8.205 ± 1.14	4.93± 0.32
Humid summer season	1	33.33± 5.66	7.88± 0.47
	2	17.15 ± 2.6	5.56 ± 0.55
	3	21.1± 3.03	6.45 ± 0.61
	4	29.06± 5.04	7.75 ± 0.45
	5	28.6± 7.70	7.53± 0 .78
	6	17.56± 3.25	5.75± 0 .5
Monsoon season	1	34.45±8.80	8.5± 0.62
	2	32.08±11.85	7.75 ± 1.15
	3	33.83±5.44	7.91 ± 0.49
	4	33.3±3.921	8.25± 0.25
	5	35.53±9.81	8.18± 0 .70
	6	36.25±6.45	7.9± 0 .57

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3.2.2. EVALUATION OF VARIOUS IMMUNO-BIOCHEMICAL PARAMETERS

3.2.2.1. Total haemocyte count

For evaluating the THC, 0.1 ml of pooled haemolymph was taken in a microfuge tube and mixed with 0.1 ml 4% NBF. After thorough mixing a drop of haemolymph –formalin mixture was placed on a clean Naeubauer slide covered with coverslip. Cells were counted using a bright field microscope and total haemocyte count was calculated by taking into account of the dilution factor using the following equation (Jalali *et al.*, 2008).

Total hemocyte count= No. of hemocyte * dilution factor * depth factor /No. of squares counted

3.2.2.2 Estimation of superoxide anion

Superoxide anion was quantified by the modified method of Song and Hsich (1994) and Arumugam *et al.* (2000). Briefly, 50 µl of haemolymph or mantle fluid was added immediately after collection into a clean microtitre plate. The mixture was then incubated at 37^0C for 1 hour. After incubation it was washed three times with phosphate buffered saline (pH -7.4). Then 0.2% NitroBlue tetrazolium (NBT) was added to all the wells and kept it at room temperature for 1 h. After the incubation absolute methanol was added for removing NBT solution. Then the sample was washed 3 times with 70% methanol and finally air dried. Finally, 120 µl 2M KOH and 140 µl DMSO were added to it to dissolve the cytoplasmic formazan. After adequate mixing, absorbance was measured at 625 nm. Super oxide anion content of samples was then calculated.

3.2.2.3. Phenoloxidase (PO) activity

PO activity was determined spectrophotometrically through the formation of a red pigment (DOPA-chrome) by oxidation of the enzyme substrate, L-dihydroxyphenylalanine (L-DOPA) (Asokan *et al.*, 1997). Briefly, 100 µl of samples

were mixed with 100 µl of phosphate buffered saline (pH 7.4), incubated at 22°C for 20 min. Following incubation, 2 ml L DOPA was added and incubated further at 22°C for 5 min. Absorbance was measured at 460 nm. PBS was taken as blank. For eliminating the effect of peroxidase, 100 µl of thiourea was added to 200 µl of samples incubated at 22°C for 20 min. Following incubation 200 µl of this solution was mixed with 2 ml of L-DOPA and incubated at 22°C for 5 min. Absorbance was measured at 460 nm. Phenol oxidase activity of samples was then calculated.

3.2.2.4. Lysozyme activity

Lysozyme activity was measured using the protocol of Ellis (1990). Briefly, 500 µl sample was mixed with 1.5 ml *Micrococcus luteus* culture (OD adjusted to 0.6-0.7) and absorbance was measured at 450 nm for 5 min in 1-min interval. Phosphate buffer (pH-7) was used as blank. Overnight culture of Micrococcus, O D maintained at 0.6- 0.7 was used. A standard curve was plotted using serial dilutions of 1ml egg white in 30 ml phosphate buffer as standard. Lysozyme activity (U/ml) was then calculated.

3.2.2.5. Glucose content

Glucose content was determined by GOD/POD method using glucose kit (Coral clinical systems). Samples (0.01 ml) were mixed with 1 ml of glucose reagent and incubated at 37°C for 10 min. Absorbance was measured at 505 nm using a spectrophotometer. A standard curve was made using serial dilutions of glucose standard (from 0.00487 mg ml^{-1} to 10 mg ml^{-1}) and distilled water as blank. Graph was plotted by taking glucose concentration (mg ml^{-1}) on the X axis and absorbance (Abs) measured on the Y axis. Glucose content present in the samples was calculated by using the following equation

Glucose content (mg/ml) = Abs of test / Abs of standard *1

3.2.2.6 Estimation of total haemolymph protein

Total haemolymph protein was estimated as per Lowry *et al.* (1951) using bovine serum albumin as standard. Briefly, 1 ml of 10% TCA (Trichloroacetic acid) was added to 0.1 ml of the samples and kept it at 4° C for 1 hour for precipitation of protein. Then the mixture was centrifuged at 5000 rpm for 5 min. The supernatant was discarded and 0.5 ml of 0.1 N NaOH was added to the pellet for dissolving tissues. 0.1 ml of this solution was taken for protein estimation. The solution(0.1ml) is mixed with 500 µl of alkaline copper sulphate reagent and incubated for 10 min at room temperature. After incubation, 50 µl of Folin's reagent was added followed by 30 min incubation in dark. Absorbance was measured at 660 nm using a spectrophotometer. In blank distilled water was used instead of sample. Protein content of samples was calculated by using the following equation

Concentration of protein (mg/ml) = (OD of test/OD of standard) * concentration of standard* dilution factor

3.2.2.7. Total albumin

Total albumin was determined by Bromocresol Blue method using albumin kit (Coral clinical systems). Briefly, 0.01 ml of samples were mixed with 1 ml of BCG reagent and incubated at room temperature for 5 min and absorbance was measured at 630 nm using a spectrophotometer. Along with this, a standard curve was plotted using serial dilutions of albumin standard (from 0.0156 g dL^{-1} to 4 g dL^{-1}). Graph was plotted by taking albumin concentration (g dL^{-1}) on the X axis and absorbance measured on the Y axis. Distilled water was used as blank. Total albumin content present in the samples was calculated by using the following equation

Albumin content (g dL^{-1}) = Absorbance of test / Absorbance of standard *4

3.2.2.8 Total cholesterol content

Cholesterol content was determined by CHOD/PAP method using a cholesterol kit (Coral clinical systems). Briefly, 0.01 ml of samples were mixed with 1 ml cholesterol reagent and incubated at 37° C for 5 min and absorbance was measured at 505 nm using a spectrophotometer. Along with this a standard curve was made using serial dilutions of cholesterol standard (from 0.39 mg $dL^{-1}$to 200 mg dL^{-1}). Graph was plotted by taking cholesterol concentration (mg dL^{-1}) on the X axis and absorbance measured on the Y axis. Distilled water was taken as blank. Total cholesterol content present in the samples was calculated by using the following equation

Cholesterol content (mg dL^{-1}) = Absorbance of test / Absorbance of standard *200

3.2.2.9 Triacylglyceride content

Triacylglyceride content was determined by GPO/PAP method using a triacylglyceride kit (Coral clinical systems). Briefly, 0.01 ml of samples were mixed with 1 ml triacylglyceride reagent. The mixture was then incubated at 37° C for 5 min and absorbance was measured at 505 nm using a spectrophotometer. Along with this a standard curve was made using serial dilutions of triacylglyceride standard (from 0.39 mg dL^{-1} to 200 mg dL^{-1}). Graph was plotted by taking triacylglyceride concentration (mg dL^{-1}) on the X axis and absorbance measured on the Y axis. Distilled water was taken as blank. Triacylglyceride content of samples was calculated by using the following equation:

Triacylglyceride content (mg dL^{-1}) = Absorbance of test / Absorbance of standard *200

3.2.2.10 Enumeration of total viable count of bacteria

Total viable count of bacteria was enumerated following the method of Hoyda *et al.* (2007). The pooled haemolymph and mantle fluid (0.01 ml) was diluted with

90 µl of sterile normal saline. This was then serially diluted using sterile normal saline up to 10^{-6} dilution. 10 microliter (µl) of each dilution was spotted on sterile Zobell Marine Agar (ZMA) and Thiosulfate Citrate bile salts sucrose (TCBS) agar plates. Each dilution was spotted in duplicates. The plates were then incubated at 30°C for 24- 48 h. After incubation, the plates with 30-300 colonies were chosen for counting and the total viable count was expressed as log $_{10}$ colony forming units (CFU) per ml of haemolymph and mantle fluid (Hovda *et al.*, 2007).

3.2.2.11. Antibacterial activity against aquatic pathogens

Antibacterial activity was determined by spot on lawn culture method (Rojo-Bezares *et al.*, 2007). Briefly, 100 µl of cultures of each aquatic pathogens (Table no. 3.1) were spread on Muller Hinton Agar (MHA) plates. 10 µl of hemolymph and mantle fluid is spotted on each plate, incubated at 30°C overnight. Zone of inhibition is noted on the next day.

3.2.2.12 Statistical Analysis

Statistical analysis was conducted with SPSS version 16.0. Relationships of parameters with respect to seasons were determined using Mann Whitney U test (P). Overall effect of season with respect to each parameter studied was carried out using Kruskal Wallis test (P). Quantitative variables were compared using Pearson's coefficient correlation.

3.2.3 CHARACTERIZATION OF *FERRITIN* GENE OF *P. viridis*

3.2.3.1. Collection of mantle tissue

Mantle tissue was collected from the animal using sterile scissors and forceps and stored at -80° C for RNA extraction.

3.2.3.2. Extraction of total RNA from the mantle tissue of Asian green-lipped mussel

RNA was extracted using Ambion RNA isolation kit. All items were cooled before RNA extraction. The frozen mantle tissue was taken out from -80°C.and homogenized by mortar and pestle. 0.6 ml of lysis buffer was added to the mortar during homogenization and grinded the fine layer of tissue. 6 µl of mercaptoethanol was also transferred to grinded tissue. Once homogenized the solution was transferred to cooled Eppendorf tubes, it was centrifuged at 12000 g for 2 min. Following centrifugation supernatant was transferred to a fresh tube, and care was taken not to contaminate the solution. 300 µl of 70% ethanol was added to the supernatant and vortexed thoroughly. Centrifuged the sample at 12000 g for 15sec at room temperature. Following centrifugation, the supernatant was removed and 350 µl of wash buffer was added to the column and centrifuged at 12000g for 15 sec. Then the column was transferred to a new collection tube and incubated at room temperature for 15 sec. 350 µl wash buffer1 was added to the column and spin at 12000 g for 15 sec. The flow through was discarded and 500 µl of wash buffer was added followed by centrifugation at 12000 g for 15 sec. The flow through was discarded. Additional centrifugation was carried out 12000 g for 1min to dry the membrane. The column was transferred to new labelled 1.5ml centrifuge tube. 25 µl of nuclease free water was added and incubated at room temperature for 1 min and centrifuged at 12000 g for 1 min. The eluted sample was stored at -80°C after quantifying the RNA using bio photometer.

3.2.3.3. Synthesis of complementary DNA

Complementary DNA was synthesized using the Thermo Scientific Revert Aid First Strand cDNA synthesis kit. The cDNA was reverse transcribed from the RNA, using 1 µl of oligo (dT) primer, 2 µl dNTP, 1 µl Reverse transcriptase, 4 µl of 5X reaction buffer, 1 µl RNAase inhibitor and 6 µl of nuclease free water.

3.2.3.4 PCR amplification of *ferritin* gene fragment

A fragment of *ferritin* gene was amplified using degenerate primers; FerrD1- and FerrD2 (Table 3.2). The optimized PCR mixture consisted of 1 µl of template DNA, 2.5 µl of reaction buffer, 0.5 µl of dNTP, 0.5 µl of forward and reverse primer and 0.25 µl of Taq polymerase. The optimized PCR programme included initial denaturation at (94°C for 5 min) followed by 35 cycles of denaturation (94°C for 30 sec), annealing (51°C for 1 min) and extension (72°C for 1. 30 min). Final extension was carried out at 72°C for 10 min.

3.2.3.5 Analysis of PCR products by Agarose gel electrophoresis

The PCR products were then analyzed on 1.5% agarose gel by gel electrophoresis. One kb DNA ladder was used as molecular weight marker. Briefly, 5 µl of DNA was mixed with 1 µl bromophenol blue and loaded onto agarose gel. The agarose gel was run at constant voltage of 80 V and current of 45A till the bromophenol blue has reached the extreme opposite side of the wells. Viewed the gel on UV transilluminator with the safety shield and photograph was taken.

3.2.3.6. Purification of *ferritin* gene fragment

PCR product was purified using a gel extraction kit (GenElute –Sigma Aldrich). 1.5% agarose gel was prepared and 100 µl of DNA with 20 µl bromophenol blue was loaded on the gel and run at 90 V for 40 min. DNA band was excised from the gel using a clean sterile razor blade after viewed in UV transilluminator. Weight of the gel slice was noted and three gel volumes of gel solubilisation solution was added to it and incubated at 55 °C for 10 min. Vortexed the gel mixture every 3 min for complete dissolution of the gel. Column was prepared prior to elution by adding a column preparation solution. It was centrifuged at 12000 rpm for 2 min. After complete dissolution of the gel 10 µl of 3 M sodium acetate was added for maintaining the pH of the solubilization buffer. Two gel volume of isopropanol was added, mixed well and 700 µl of this mixture was loaded into the binding column that

is assembled in the collection tube. It was centrifuged at 12000 rpm for 2 min, discarded the flow through and repeated the steps until complete mixture is loaded into the column. Then 700 µl of wash buffer was added and centrifuged at 12000 rpm for 2 min. Additional centrifugation was carried out to remove the remaining residues present in the column. After that the column was transferred to a new collection tube and 25 µl of elution buffer was added, incubated for 5 min at 37° C and centrifuged at 12000 rpm for 5 min. Eluted product was stored at -20° C.

3.2.3.7 Sequencing and sequence analysis

The PCR products were sequenced at Agrigenome, Kochi and the sequence were then subjected to BLAST search (NCBI) for the confirmation of the amplicon.

3.2.3.8 Full gene characterization of *ferritin* gene by RACE-PCR

3' and 5' RACE PCR was done using 5'/3' RACE kit, 2nd generation (Sigma Aldrich)

3.2.3.8.1 3'RACE of *ferritin* gene

3.2.3.8.1.1 Total RNA extraction from mantle tissue

Total RNA was extracted from 100 mg mantle tissue using the same procedure described in 3.2.35.1

3.2.3.8.1.2 Synthesis of 3' first strand cDNA using oligo dT anchor primer

First strand cDNA was synthesised using 5'-3' RACE kit, 2nd generation (Sigma Aldrich). The mixture consisted of 2 µl of total RNA, 1 µl of RNAase inhibitor, 4 µl of cDNA synthesis buffer,2 µl of dNTP,1 µl of oligo dT anchor primer,1 µl of transcriptor RT. Incubated the mix at 55° C for 60 min. Then incubated it at 85°C for 5min.

3.2.3.8.1.3 PCR using gene specific primer

The 3' end of *ferritin* gene was then amplified using a gene specific primer designed during the present study (Table 3.2). PCR conditions and mixture was optimized using standard protocol (Sambrook and Russel, 2003). Final optimized PCR mixture consisted of 1 µl of cDNA, 2.5 µl of reaction buffer, 1 µl of dNTP, 1 µl each of forward and reverse primer, 18.25 µl nuclease free water and 0.25 µl of *Taq* DNA polymerase. The optimized thermal conditions included initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min) and extension (72°C for 1. 30 min). Final extension was carried out at 72°C for 10 min. The PCR products were then characterized by 1% agarose gel electrophoresis. One kb DNA ladder was used as molecular weight marker. After electrophoresis the gel was visualized in Gel documentation system and gel picture was taken.

3.2.3.8.1.4 Gel purification of 3' end of ferritin gene

PCR product was purified using a gel extraction kit (GenElute- Sigma Aldrich) following the procedure described in 3.2.3.6.

3.2.3.8.1.5 Cloning of 3' end of ferritin gene in pTZ57R/T vector

3.2.3.8.1.5.1 Ligation of 3' end of ferritin gene in pTZ57R/T vector

Firstly, the purified PCR product was ligated in pTZ57R/T vector using ligase enzyme. The ligation mix consisted of 1 µl of Vector, 2 µl of 5X ligation buffer, 2 µl PCR product, 4 µl Nuclease free water and 1 µl of ligase enzyme. The reaction mix was incubated at 4°C overnight.

3.2.3.8.1.5.2 Preparation of competent E. coli DH5α cells

Competent cells were prepared following the protocol of Sambrook and Russel, 2003. Briefly, the E. coli DH5α cells from glycerol stock were streaked on LB agar plates

and incubated at 37°C overnight. A single colony of E. coli DH5α was then inoculated in 5 ml LB broth and grown overnight at 37° C. On the next day 1 ml of this overnight culture was added to 100 ml LB broth and grown at 37°C for 3 hours. Following incubation, the cells were placed on ice for 10 min. Then cells were collected by centrifugation at 6000 rpm for 3 min at 4° C. The pellet was then suspended in 10 ml cold 0.1 M $CaCl_2$. Special care was taken while suspending the pellet to avoid mechanical disruption of the cell. It was again incubated on ice for 20 min. After incubation it was centrifuged at 6000 rpm for 3 min, discarded the supernatant and gently suspended the cells on 5 ml cold 0.1 M $CaCl_2$ /15 % glycerol and distributed it in 1.5 ml microfuge tubes (300 µl in each tube).

3.2.3.8.1.5.3 Transformation of ligated plasmid into competent E. coli DH5α cells

Transformation was done by heat shock method using 100 µl of competent *E. coli* DH5α cells (Sambrook and Russel, 2003). Briefly, 100 µl of frozen competent cells were thawed on ice for half an hour. To this 5 µl of ligation mix was added and incubated on ice for 30 min. After that heat shock was given at 42° C for 2 min and immediately chilled on ice. Then 900 µl of Luria Bertani broth was added to it and kept in a shaker incubator for 30 min at 37°C. Following incubation, transformed *E. coli* culture was plated on LB agar containing ampicillin (100mg/ml), IPTG (24 mg/ml) and X Gal (20mg/ml). The plates were incubated at 37°C overnight.

3.2.3.8.1.5.4 Selection of recombinant clones

The white colonies obtained in LB plates after incubation were selected and suspended in 100 µl of nuclease free water. 10 µl of this was used for crude DNA preparation. Colony PCR was done using this crude DNA with gene specific primer and PCR anchor primer. 90 µl of positive colony suspensions were inoculated in 10 ml LB broth containing 100 mg/ml ampicillin and grown overnight at 37°C and plasmid DNA was isolated on the next day using Genejet plasmid isolation kit (Thermfischer Scientific) following the manufacturer's protocol. Briefly, the

overnight culture was centrifuged at 10000 rpm for 10 min, supernatant was discarded and pellet was suspended in 300 µl of resuspension solution (P1). Then 300 µl of lysis buffer (P2) was added and mixed thoroughly by inverting the tubes for 4-5 times. It was then incubated at 37° C for 3 min. Following incubation, 300 µl of neutralization solution (P3) was added and kept on ice for 15 min. After that it was mixed and centrifuged at 13000 rpm for 10 min. Supernatant was then transferred to a Genejet spin column attached to a collection tube and centrifuged at 13000 rpm for 1 min. Flow through was discarded and 500 µl of wash buffer was added to it. Then it was centrifuged at 13000 rpm for 1 min. Repeated the washing step one more time to completely remove all residues in column. Additional centrifuge was done at 13000 rpm for 1 min to remove the residual wash buffer. Column was then transferred to a new collection tube and 25 µl of elution buffer was added, incubated at 37° C for 2 min and centrifuged at 13000 rpm for 2 min. Column was discarded and purified plasmid DNA was stored at -20° C.

3.2.3.8.1.5.5 PCR using M13 primers

The specific insert flanking M13 primer sequences were amplified using M13 forward and M13 reverse primers. 1 µl of isolated plasmid is used as template here. The reaction mixture consisted of 1 µl of plasmid DNA (1:10 dilution), 2.5 µl of reaction buffer, 0.5 µl of dNTP, 1 µl of M13 forward and reverse primers, 18.75 µl nuclease free water and 0.25 µl of *Taq* polymerase. The thermal conditions included initial denaturation at (94°C for 5 min) followed by 35 cycles of denaturation (94°C for 1 min), annealing (52°C for 1 min) and extension (72°C for 1. 30 min). Final extension was carried out at 72°C for 10 min. The PCR products were then characterized by 1.5% agarose gel electrophoresis.

3.2.3.8.1.5.6 Sequencing and sequence analysis

The PCR product obtained was sent for sequencing with M13 forward primer. (Agrigenome, Kochi). The obtained sequences were then subjected to BLAST search (NCBI) for the confirmation of the amplicon.

3.2.3.8.2. 5'RACE of *ferritin* gene

3.2.3.8.2.1 RNA extraction from mantle tissue

Total RNA was isolated from 100 mg mantle tissue using the same procedure described in 3.2.4.1.

3.2.3.8.2.2 First strand cDNA synthesis using gene specific primer

First strand cDNA was synthesized using gene specific primer namely, fer 5'cDNA designed in the present study (Table 3.2). The mixture consisted of 2 µl of total RNA, 1 µl of RNAase inhibitor, 4 µl of cDNA synthesis buffer, 2 µl of dNTP, 1 µl of fer 5'cDNA primer,1µl of transcriptor RT. Incubated the mix at 55°C for 60 min followed at 85°C for 5 min.

3.2.3.8.2.3 Purification of cDNA

cDNA was purified using high pure PCR product purification kit (Sigma Aldrich). Firstly 100 µl of binding buffer was added to 20 µl of the first strand cDNA, mixed well and loaded into the column attached to a collection tube. It was centrifuged at 8000 g for 1 min. Flow through was discarded and 500 µl of wash buffer was added. It was then centrifuged at 8000 g for 1 min and flow through was again discarded. 200 µl of wash buffer was then added to the same column, centrifuged at 13000 g for 2 min and the flow through was discarded. Column was then transferred to a new collection tube and cDNA was eluted in 25 µl of elution buffer.

3.2.3.8.2.4 dA tailing at 3' end

Homopolymeric tailing reaction mix consisted of 19 µl of purified cDNA, 2.5 µl of reaction buffer and 2.5 µl of dATP. Firstly, the mixture was incubated at 94°C for 3 min. Then it was immediately chilled on ice and 1 µl of terminal transferase enzyme was added, vortexed and spined down briefly. It was then incubated at 37°C for 30 min. After that the reaction was stopped by heating at 70°C for 10 min. The tailed cDNA was spin down and placed on ice.

3.2.3.8.2.5. PCR using nested gene specific primer and oligodT anchor primer

The reaction mixture consisted of 2.5 µl of dA tailed cDNA, 2.5 µl of reaction buffer,0.5 µl of dNTP, 0.5 µl of dTanchor primer, 0.625 µl of Fer 5 rev primer, 18.75 µl nuclease free water and 0.25 µl of *pfu* polymerase. The thermal conditions included initial denaturation at (95°C for 2 min) followed by 10 cycles of denaturation (95°C for 15 sec), annealing (44°C for 30 sec) and extension (72°C for 40 sec) and 15 cycles of denaturation (95°C for 15 sec), annealing (44°C for 30 sec) and extension (72°C for 40 sec). Here extension was increased by 20 secs in every cycle from the 11^{th} cycle onwards. Final extension was carried out at 72°C for 10 min. The PCR products were then characterized by 1.5% agarose gel electrophoresis using100 bp DNA ladder as molecular weight marker. After electrophoresis the gel was visualized in Gel documentation system and picture was taken.

3.2.3.8.2.6 Gel purification

PCR product was purified using a gel extraction kit (GenElute –Sigma Aldrich) following the procedure described in 3.2.4.4.

3.2.3.8.2.7 Cloning of 5' end of ferritin gene in pTZ57R/T vector

Cloning of 5' end of *ferritin* gene was done following the same method described in 3.2.3.8.1.5. Colony PCR was done using dTanchor primer and Fer 5 Rev (Table 3.2) The PCR product obtained after amplification from isolated plasmid was given for

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sequencing with M13 reverse primer (Table 3.2). The obtained sequences were then subjected to BLAST search (NCBI) for the confirmation of the amplicon.

3.2.3.8.3 Sequence analysis of complete *ferritin* gene

The obtained sequence data were edited and assembled using Editseq (DNASTAR, Lasergene, v6.0, USA). The sequence was then compared with those in GenBank database, National Centre for Biotechnology Information (NCBI) using nucleotide BLAST with default parameters and closest relative sequences were retrieved. The DNA sequences obtained were then converted into amino acid sequences and then compared with those in GenBank database, NCBI using BLASTP with default parameters. Sequence alignment with the relative sequences in NCBI was performed using CLUSTAL V and the aligned data set was used as input for phylogenetic analysis using DNASTAR, Lasergene, v6.0, USA. Tree topology was estimated by bootstrap analyses based on 1000 replicates, and phylogenetic tree was constructed using neighbor-joining (NJ) method. Further, ORF region was predicted using ORF Finder. Conserved domain search from NCBI was used to identify domain region. Physicochemical properties for amino acid sequence was predicted using ProtParam.

RESULTS

4. RESULTS

The results of the work entitled "Evaluation of immuno- biochemical attributes of Asian green lipped mussel (*Perna viridis*) and characterization of selected immune related gene" conducted at the ICAR- Central Marine Fisheries Research Institute, Kochi during 2018-2019 are presented in this chapter.

4.1 SAMPLE COLLECTION

Samples were collected from different areas of Cochin coastal line during three seasons namely, cool dry season (November-February), humid summer season March-May) and Monsoon season (June-September) (Plate 4.1). Average condition index of mussels in all three seasons are calculated and the values are 0.97709±0.13 in cool dry season (November-February), 5.5477±0.93 in humid summer season March-May) and 8.321±0.25 in monsoon season (June-September).

4.1.1 Physicochemical parameters:

All physicochemical parameters during each season were recorded and shown in Table no. 4.1

Table 4.1 Physicochemical parameters of water collected from the sampling site

Seasons	Surface temperature (°C)	pH	Salinity (ppt)	Dissolved oxygen (ppm)
Cool dry season	28.425±0.154	7.2	30	5.5
Humid summer season	29.666±0.240	7.3	32	5.1
Monsoon season	27.45±0.3175	7.5	25	5.4

4.2 DETAILS OF HEMOLYMPH, MANTLE FLUID AND MANTLE TISSUE

About 1 ml of haemolymph and mantle fluid were collected from apparently health mussels in all three seasons studied. Haemolymph and mantle fluid of six animals were pooled to reduce the individual variation. It was then stored at 4° C for doing further study (Plate 4.2). Mantle tissue was collected from mussels and stored in sterile vials free of RNase and stored at -80° C for molecular study.

Plate 4.1 Sample collection site map

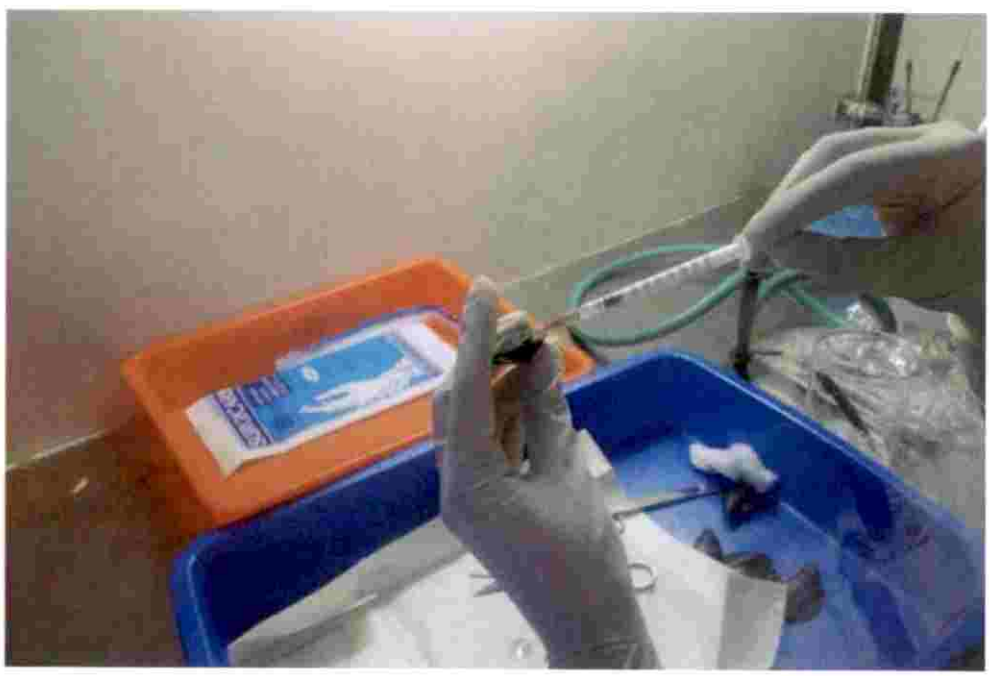

Plate 4.2 Haemolymph and Mantle fluid collection

4.3 EVALUATION OF IMMUNO-BIOCHEMICAL ATTRIBUTES IN HAEMOLYMPH OF *P. VIRIDIS*

A total of 11 immuno-biochemical parameters were evaluated in haemolymph of *P. viridis* during three seasons and tabulated in Table 4.2.

4.3.1 Immunological parameters

4.3.1.1 Total Hemocyte Count (THC)

Hemocytes were counted in Naeubauer chamber using a bright field microscope (Plate 4.3). Total hemocyte count of *P. viridis* was comparable between the three seasons studied, having no significant difference between the seasons ($p>0.05$) (Fig.4.1). However, the average hemocyte count was higher (78200 cells/ml) in cool dry season and lowest (70600 cells/ml) in humid summer season. The total hemocyte count were varied from $2.8 * 10^3$ cells/ml to $1.2 * 10^4$ cells/ml in cool dry season with a gradual decrease to humid summer season ($2.6 * 10^3$ cells/ml to $9.3 * 10^3$ cells/ml) following an increasing trend towards monsoon season ($3.8 * 10^3$ cells/ml to $1.5 * 10^4$ cells/ml (Fig.4.2a & 4.2b).

4.3.1.2 Phenoloxidase Activity

Phenoloxidase activity ranged from 0.0015 units/min/mg protein to 0.006 units/min/mg protein annually (Fig.4.3a). However, significant difference in phenoloxidase activity was observed between each seasons ($P<0.05$) (Fig.4.4a). Average phenoloxidase activity was higher (0.0041 units/min/mg protein) in monsoon season and lowest (0.0015 units/min/mg protein) in humid summer season (Fig4.4a). The circannual phenoloxidase activity was showing a gradual decrement from cool dry season to humid summer seasons following a steep increment towards the monsoon season (Fig.4.5a).

4.3.1.3 Superoxide anion generation

Superoxide anion production in haemolymph varied from 0.063-0.1735 OD/mg protein in cool dry season, 0.0315-0.162 OD/mg protein in humid summer and 0.0135-0.148 OD/mg protein in monsoon season (Fig. 4.3b). It was observed that there is significant difference between superoxide anion production of all three seasons ($p<0.05$); however, the superoxide anion production was not differed significantly between the humid summer season and monsoon season ($p>0.05$) (Fig.4.4b). Highest superoxide anion production (0.1336 OD/mg protein) was observed in cool dry season and lowest was observed in humid summer season (0.0654). The circannual superoxide anion production showed a gradual decrement from cool dry season to humid summer and toward monsoon season also (Fig.4.5b).

4.3.1.4 Lysozyme Activity

No lysozyme activity was observed in haemolymph in any of the seasons. According to the principle, a decrease in absorbance in one-minute interval indicated the lysozyme activity, but here absorbance value was observed increasing when measured in one-minute interval.

4.3.2 Biochemical parameters

4.3.2.1Glucose level

Glucose level varied from 0.023-0.067 mg/ml in cool dry season, 0.034-0.162 mg/ml in humid summer season and 0.014-0.072 mg/ml in monsoon with no significant difference ($p>0.05$) observed between all three seasons (Fig.4.6a). It was found that, average glucose value was highest in humid summer season (0.0533mg/ml) and lowest in cool dry season (0.0406 mg/ml). However, the glucose level for all these seasons were comparable between each other (Fig. 4.8a). The seasonal trend for glucose had shown a sharp increase in humid summer followed by a decrease in monsoon season (Fig.4.9a). In the cool dry season glucose has showed a

positive correlation with condition index. But in the other two seasons no such correlation was observed.

4.3.2.1Total protein

Level of haemolymph protein varied from 1.18-2.275 mg/ml in cool dry season, 0.53-2.32 mg/ml in humid summer season and 0.364-1.20mg/ml in monsoon season and a significant difference in the protein level was observed between all three seasons ($p<0.05$) (Fig. 4.6b). However, no significant difference in protein level was observed between the first two season (cool dry season and humid summer season) ($p>0.05$). But a significant difference was observed between cool dry and monsoon season($p<0.05$). Humid summer and monsoon season also showed a significant difference ($p<0.05$). Average protein value was higher (1.73mg/ml) in cool dry season and least (0.623mg/ml) during monsoon season (Fig. 4.8b). Circannual trend for haemolymph protein showed a gradual decrease from cool dry season to humid summer season followed by a sharp decrease in monsoon season (Fig. 4.9b).

4.3.2.3 Albumin level

Trace level (below detectable range of assay) of albumin was observed in haemolymph which was approximated to null throughout the assay.

4.3.2.4 Cholestrol level

Level of haemolymph cholesterol varied from 22.9-27.05 mg/dl in cool dry season, 1.8-9.63 mg/dl in humid summer season and 0.519-6.23 mg/dl in monsoon season with a significant difference of probability value $p<0.05$ (Fig. 4.7a). However, no significant difference was observed between humid summer and monsoon season ($p>0.05$). Average cholesterol value was higher (24.29mg/dl) in cool dry season and least (3.446 mg/dl) during monsoon season (Fig. 4.8c). Circannual trend for haemolymph cholesterol showed a steep decrease from cool dry season to humid summer season followed by a gradual decrease in monsoon season (Fig. 4.9c).

Cholesterol content of haemolymph in humid summer season showed a positive correlation with total hemocyte count.

4.3.2.5 Triacylglyceride level

It was observed that the haemolymph triacylglyceride varied from 23.76-28.75mg/dl in cool dry season, 25.39-32.30 mg/dl in humid summer season and 5.99-19.6 mg/dl in monsoon season with a significant difference between all three seasons studied ($p<0.05$) (Fig. 4.7b). Triacylglyceride of haemolymph showed individual significant difference in between each seasons. Highest triacylglyceride level was observed in humid summer season (30.47mg/dl) and least value is witnessed in monsoon season (15.13mg/dl) (Fig. 4.8d). Circannual pattern of triacylglyceride showed a gradual increment from cool dry season to humid summer season and a sharp decrease in monsoon season (Fig. 4.9d).

4.3.3 Microbiological parameters

4.3.3.1 Total viable count of bacteria

Total viable count of bacteria was enumerated in ZMA (Plate 4.4) and Total vibrio count was enumerated in TCBS (Plate 4.5). Total viable count of bacteria (TVC) in haemolymph varied from 2.60-3.477 $\log_{10}$ CFU/ml in cool dry season, 2.602-4.632 $\log_{10}$ CFU/ml in humid summer season and 2-4 632 $\log_{10}$ CFU/ml in monsoon season. It was observed that there is no significant difference between TVC of all three seasons ($p>0.05$). No significant difference was observed between any of the seasons. When comparing the TVC of these three seasons highest value was obtained in humid summer season and lowest value was observed in cool dry season (Fig 4.10a). The total viable count of Vibrio spp. showed a significant difference circanually ($p<0.05$). Highest count was observed in humid summer season and lowest was observed in cool dry season. A significant difference was observed between cool dry season and humid summer season($p<0.05$) (Fig.4. 10b).

4.3.3.2 Antibacterial activity against aquatic pathogens

No antibacterial activity was observed in haemolymph collected in humid summer season and monsoon season. While haemolymph collected in cool dry season showed antibacterial activity against *Vibrio parahemolyticus* with a zone diameter of 4 mm (Plate 4.6).

Table 4 .2 Average values of all parameters studied in haemolymph during all three seasons

	Cool dry season				Humid summer season				Monsoon season			
Parameters	Mean	SD	SE	Normal Range	Mean	SD	SE	Normal Range	Mean	SD	SE	Normal Range
Total hemocyte count(cells/ml)	78213	44722	18257	$2.8*10^3$ - $1.2*10^4$	70550	24360	9945.62	$2.6*10^3$-$9.4*10^3$	75330	39515	16130	$3.8*10^3$-$1.52*10^4$
Phenoloxidase activity(units/min/mg protein)	0.002	0	0	0.002	0.0015	0	0	0.0015	0.0040	0.0018	7.3786	0.002-0.006
Superoxide anion generation(OD/mg protein)	0.1366	0.0570	0.0232	0.063-0.1735	0.075	0.455	0.0185	0.0315-0.162	0.0654	0.649	0.0264	0.0135-0.148
Glucose(mg/ml)	0.0406	0.2079	0.0084	0.023-0.067	0.0533	0.2199	0.008	0.034-0.162	0.0468	0.0246	0.01	0.014-0.072
Total protein(mg/ml)	1.727	0.599	0.244	1.18-2.275	1.391	0.834	0.340	0.53-2.32	0.623	0.3262	0.133	0.364-1.20
Cholesterol(mg/dl)	24.296	2.132	0.8706	22.9-27.05	6.54	3.15	1.28	1.8-9.63	3.446	2.556	1.043	0.519-6.23
Triacylglycerides(mg/dl)	26.165	2.63	1.075	23.76-28.75	30.47	3.948	1.61	25.39-32.30	15.13	5.004	2.042	5.99-19.6
TVC in ZMA	3.002	0.3954	0.1014	2.60-3.477	3.527	0.766	0.312	2.602-4.632	3.225	0.839	0.342	2-4
Total Vibrio count	2.0070	0.6527	0.266	1.176-2.544	3.023	0.407	0.182	2.602-3.162	2.450	0.693	0.283	1.3-3

4.4 EVALUATION OF IMMUNO-BIOCHEMICAL ATTRIBUTES IN MANTLE FLUID

Table 4.3 shows the summary statistics for different immuno-biochemical variation in mantle fluid with respect to the season studied.

4.4.1 Immunological parameters

4.4.1.1Phenoloxidase activity

Phenoloxidase activity ranged from 0.0015 units/min/mg protein to 0.0045 units/min/mg protein in cool dry season, 0.0025 units/min/mg protein to 0.0065 units/min/mg protein in humid summer season and 0.006 units/min/mg protein to 0.007 units/min/mg protein in monsoon season (Fig. 4.3c). However, a significant difference in phenoloxidase activity was observed between all three seasons ($P<0.05$). Average mantle fluid phenoloxidase activity was significantly differed between cool dry season- humid summer season and cool dry season-monsoon season (Fig. 4.4a). But no significant difference was observed between humid summer- monsoon season. Highest value was observed in monsoon season (0.0063 units/min/mg protein) and lowest (0.003 units/min/mg protein) in cool dry season. The circannual phenoloxidase activity in mantle fluid was showing a gradual increment from cool dry season to monsoon season (Fig 4.5a).

4.4.1.2 Superoxide anion generation

Superoxide anion production in mantle fluid varied from 0.06 OD/mg protein to 0.094 OD/mg protein in cool dry season, 0.0265 OD/mg protein to 0.0870 OD/mg protein in humid summer season and 0.0815 OD/mg protein to 0.1845 OD/mg protein in monsoon season (Fig. 4.3d). It was observed that there is a significant difference between superoxide anion production between first season (cool dry season), second (humid summer season) and third season (monsoon season) ($p<0.05$), whereas the superoxide anion production in haemolymph between humid summer season and

monsoon season showed no significant difference ($p>0.05$). Highest superoxide anion production (0.1114 OD/mg protein) was observed in monsoon season and lowest was observed in humid summer season (0.0553OD/mg protein) (Fig. 4.4b). The circannual superoxide anion production showed a gradual decrement from cool dry season to humid summer and a sharp increment towards monsoon (Fig.4.5b).

4.4.1.3 Lysozyme activity

Like haemolymph, no lysozyme activity was observed in mantle fluid in any of the seasons studied.

4.4.2 Biochemical parameters

4.4.2.1 Glucose level

Glucose level in mantle fluid varied from 0.025 mg/ml to 0.0320 mg/ml in cool dry season, 0.040 mg/ml to 0.110 mg/ml in humid summer season and 0.028 mg/ml to 0.059 mg/ml in monsoon season with significant difference ($p<0.05$) observed between all three seasons (Fig.4.6c). It was found that, average glucose value was highest in humid summer season (0.067 mg/ml) and lowest in cool dry season (0.0285mg/ml) (Fig.4.8a). The seasonal trend for glucose had shown a sharp increase in humid summer followed by a decrease in monsoon season (Fig.4.9a).

4.4.2.2 Total protein

Level of mantle fluid protein varied from 0.489 mg/ml to 0.568mg/ml in cool dry season, 0.52 mg/ml to 2.21 mg/ml in humid summer season and 0.126 mg/ml to 0.5630 mg/ml in monsoon season (Fig.4.6d). A significant difference in the protein level was observed between all three seasons ($p<0.05$). A significant difference in varied protein level was observed between cool dry season and humid summer season, humid summer and monsoon season, cool dry season and monsoon season with a probability value $p<0.05$ (Fig.4.8b). Average protein value was higher (1.064 mg/ml) in humid summer season and least (0.319mg/ml) in monsoon season.

Circannual trend for mantle fluid protein showed a sharp increase from cool dry season to humid summer season followed by a sharp decrease in monsoon season (Fig.4.9b).

4.4.2.3 Albumin level

However, trace level (below detectable range of assay) of albumin was observed in mantle fluid which was approximated to null throughout the assay.

4.4.2.4 Cholesterol level

Level of mantle fluid cholesterol varied from 2.35 mg/dl to 4.7 mg/dl in cool dry season, 1.24 mg/dl to 6.62 mg/dl in humid summer season and 0.02 mg/dl to 1.74 mg/dl in monsoon season (Fig.4.7c). A significant difference was observed in cholesterol level between the cool dry season, humid summer season and monsoon season with a probability value $p<0.05$. But no significant difference was observed during cool dry season and humid summer season. Average cholesterol value was similar in cool dry season and humid summer season (3.5 mg/dl) while least (0.625 mg/dl) was observed in monsoon(Fig.4.8c). Circannual trend for mantle fluid cholesterol showed a steep decrease from humid summer season followed to monsoon season (Fig.4.9c).

4.4.2.5 Triacylglyceride level

It was observed that the triacylglyceride content in mantle fluid varied from 12.73 mg/dl to 13.33 mg/dl in cool dry season, 2.89 mg/dl to 14.03 mg/dl in humid summer and 5.17 mg/dl to 12.8 mg/dl in monsoon season (Fig.4.7d) with a significant difference between all three seasons studied ($p<0.05$); whereas there is no significant difference between humid summer season and monsoon season. Highest triacylglyceride level was observed in cool dry season (13.31 mg/dl) and least value is witnessed in humid summer season (8.24mg/dl) (Fig.4.8d). Circannual pattern of

triacylglyceride showed a gradual decrease from cool dry season to humid summer season and a gradual increase in monsoon season (Fig.4.9d).

4.4.3 Microbiological parameters in mantle fluid

4.4.3.1 Total viable count of bacteria

Total viable count of bacteria in mantle fluid varied from 2.60 $\log_{10}$ CFU/ml to 3.47 $\log_{10}$ CFU/ml in cool dry season, 3.04 $\log_{10}$ CFU/ml to 4.20 $\log_{10}$ CFU/ml in humid summer season and 2.778 $\log_{10}$ CFU/ml to 4 $\log_{10}$ CFU/ml in monsoon season. It was observed that there is no significant difference between TVC of all three seasons ($p>0.05$). No individual significant difference was observed between all the seasons. When comparing the TVC of these three seasons highest value was obtained in humid summer season (3.62 $\log_{10}$ CFU/ml) and lowest value was observed in cool dry season (3.002 $\log_{10}$ CFU/ml) (Fig 4.10a). The total viable count of *Vibrio* spp. showed a significant difference circanually ($p<0.05$). Highest count was observed in humid summer season (3.145 $\log_{10}$ CFU/ml) and lowest was observed in cool dry season (1.492 $\log_{10}$ CFU/ml). A significant difference was observed between all the three seasons studied (Fig. 4.10b).

4.4.3.2 Antibacterial activity against aquatic pathogens

No antibacterial activity was observed in mantle fluid collected in cool dry season, humid summer season and monsoon season.

Table 4.3 Average values of all parameters studied in mantle fluid during all three seasons

	Cool dry season				Humid summer season				Monsoon season			
Parameters	Mean	SD	SE	Normal Range	Mean	SD	SE	Normal Range	Mean	SD	SE	Normal Range
Phenoloxidase activity(units/ min/mg protein)	0.003	0.0164	0.00670	0.0015-0.0045	0.05	00.0019	0.0079	0.0015-0.0065	0.0063	0.00051	0.002	0.006-0.007
Superoxide anion generation(OD/mg protein)	0.713	0.017	0.0071	0.06-0.094	0.0558	0.0297	0.0122	0.0265-0.0870	0.1148	0.0415	0.0169	0.0815-0.1845
Glucose(mg/ml)	0.0285	0.00383	0.0015	0.025-0.0320	0.067	0.299	0.0122	0.043-0.110	0.0379	0.0137	0.0056	0.028-0.059
Total protein(mg/ml)	0.5285	0.0432	0.0176	0.489-0.568	1.068	0.633	0.2587	0.52-2.21	0.319	0.1598	0.0056	0.126-0.563
Cholesterol(mg/dl)	3.525	1.287	0.525	2.35-4.7	3.585	2.321	0.9479	1.24-6.62	0.6276	0.8621	0.3519	0.02-1.74
Triacylglycerides(mg/dl)	13.126	0.315	0.1285	12.72-13.33	8.243	4.89	1.998	2.89-14.03	10.62	2.98	1.219	5.17-12.8
TVC in ZMA	3.002	0.395	0.1514	2.6-3.47	3.630	0.551	0.2088	3.044-4.20	3.421	0.480	0.196	2-778-4
Total Vibrio count	1.492	0.092	0.0375	1.39-1.602	3.145	0.5506	0.246	2.477-3.792	2.446	0.368	0.150	2-3

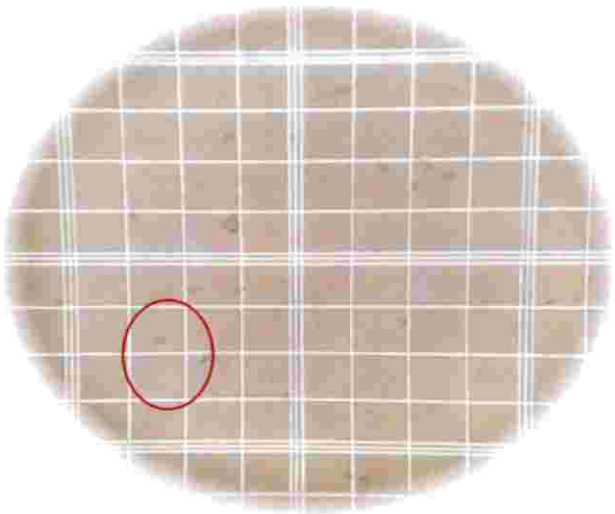

Plate 4.3 Figure showing hemocyte in Naeubauer chamber

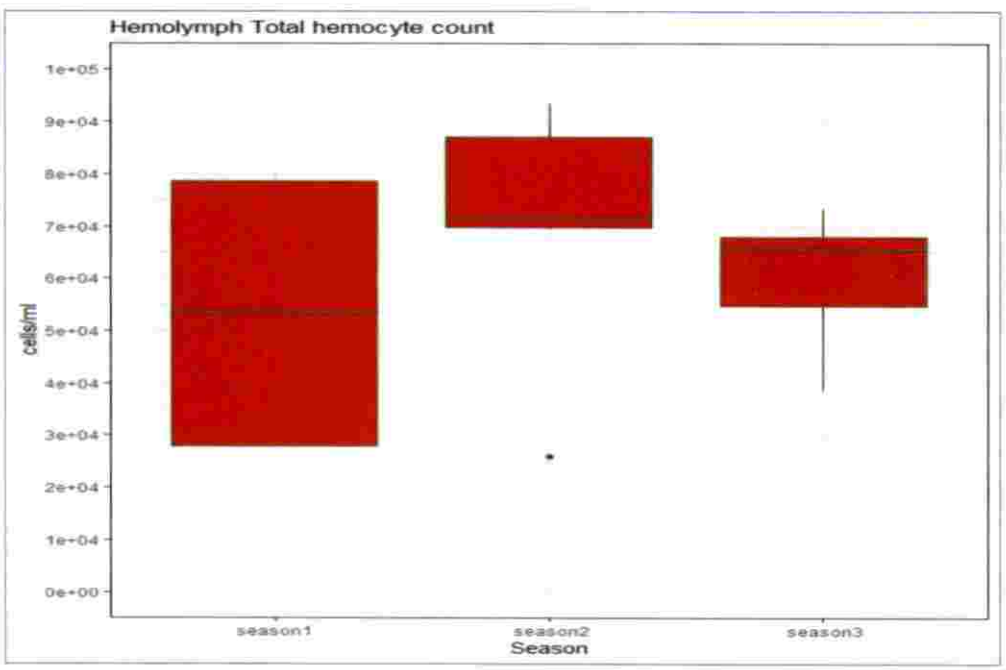

Fig 4.1 Boxplot showing haemolymph Total Hemocyte Count (THC)

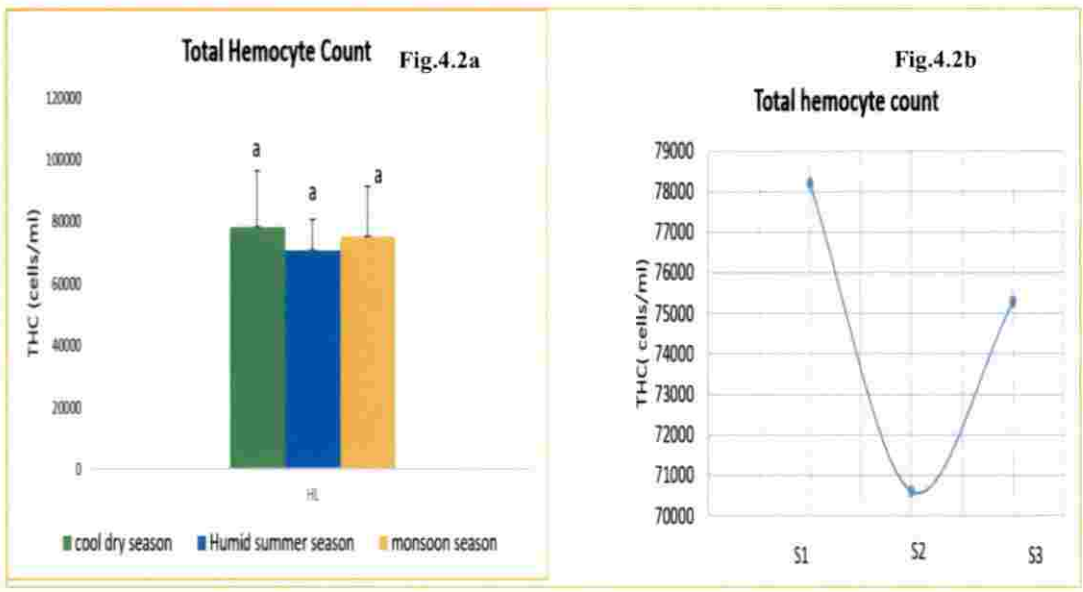

Fig. 4.2 Clustered bar diagram and trend line graph showing total hemocyte count in haemolymph

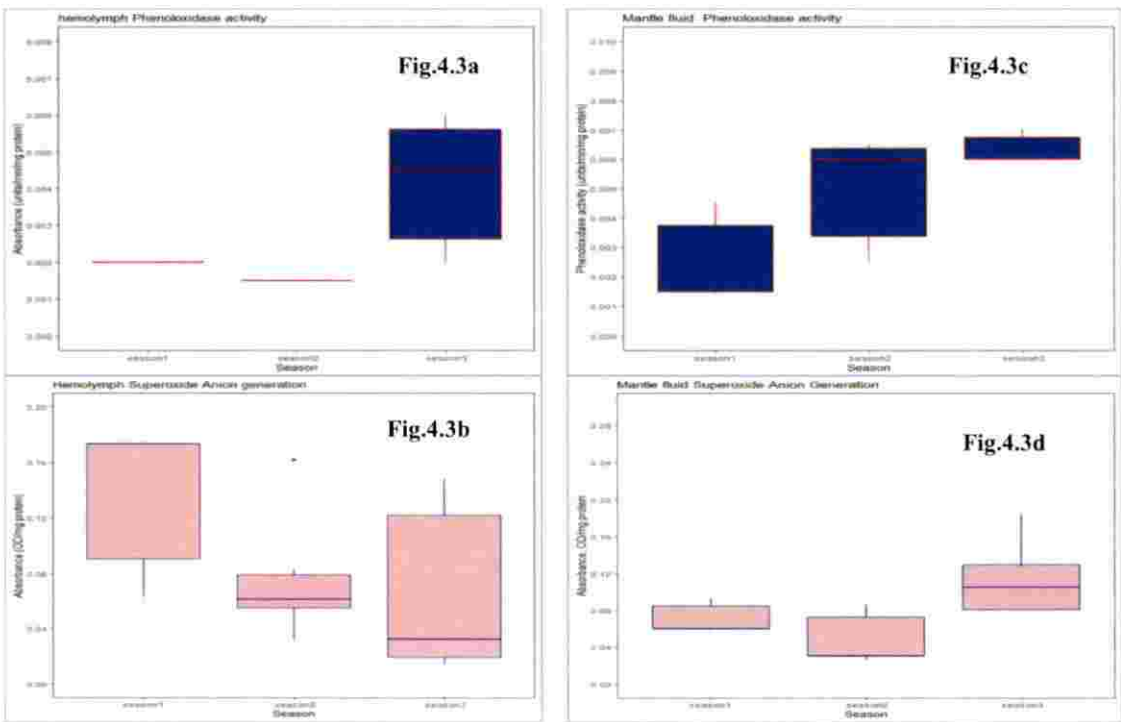

Fig 4.3 Boxplot showing phenoloxidase activity and superoxide anion generation in haemolymph and mantle fluid

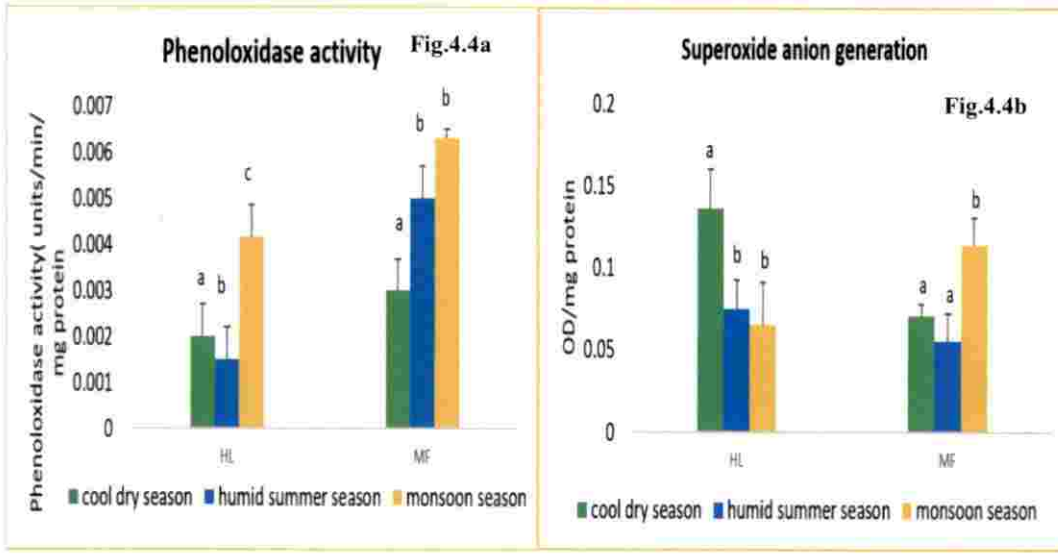

Fig. 4.4 Clustered bar diagram showing phenoloxidase activity and superoxide anion generation in haemolymph and mantle fluid

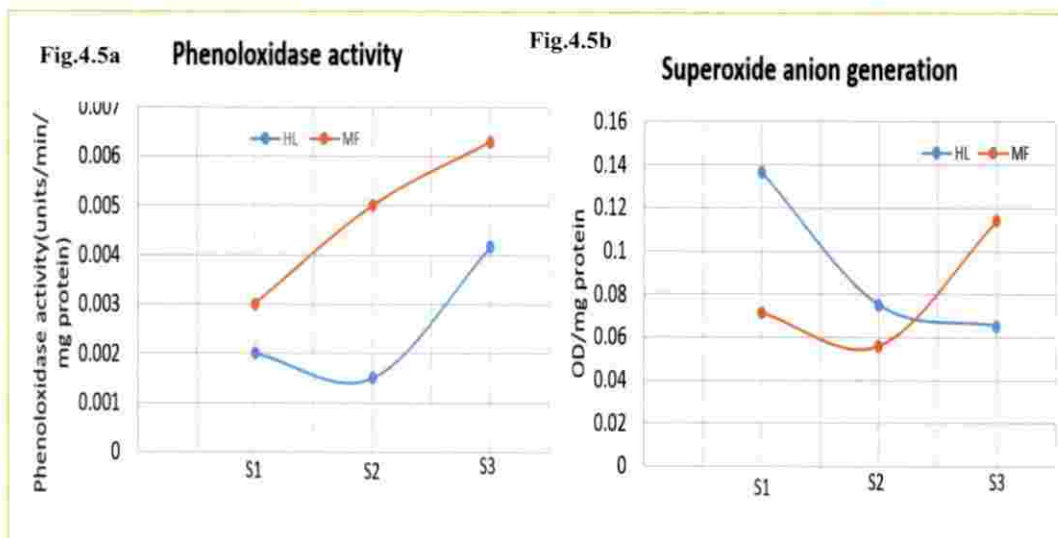

Fig. 4.5 Trend line graph showing phenoloxidase activity and superoxide anion generation in haemolymph and mantle fluid

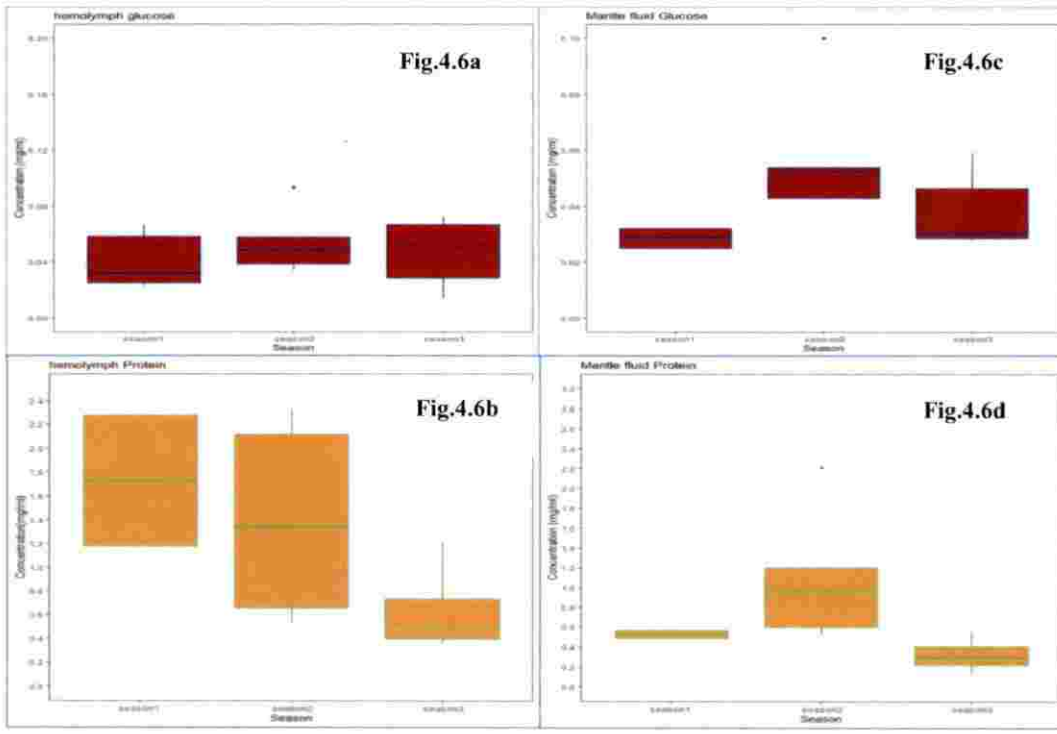

Fig. 4.6 Boxplot showing glucose level and total protein in haemolymph and mantle fluid

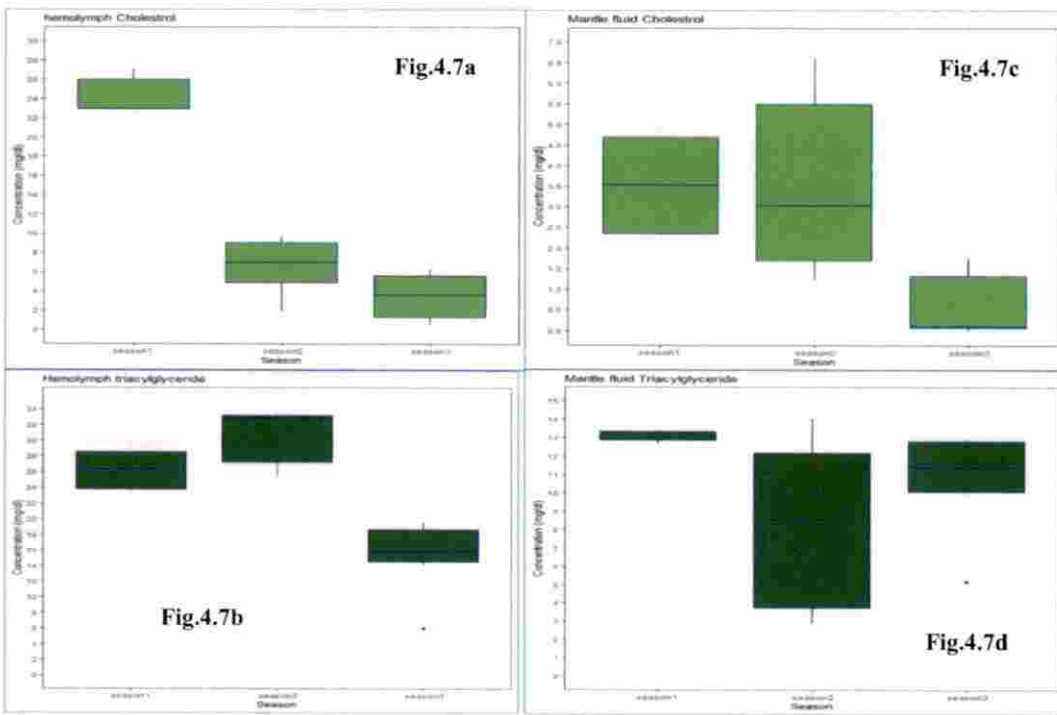

Fig. 4.7 Boxplot showing cholesterol and triacylglyceride in haemolymph and mantle fluid

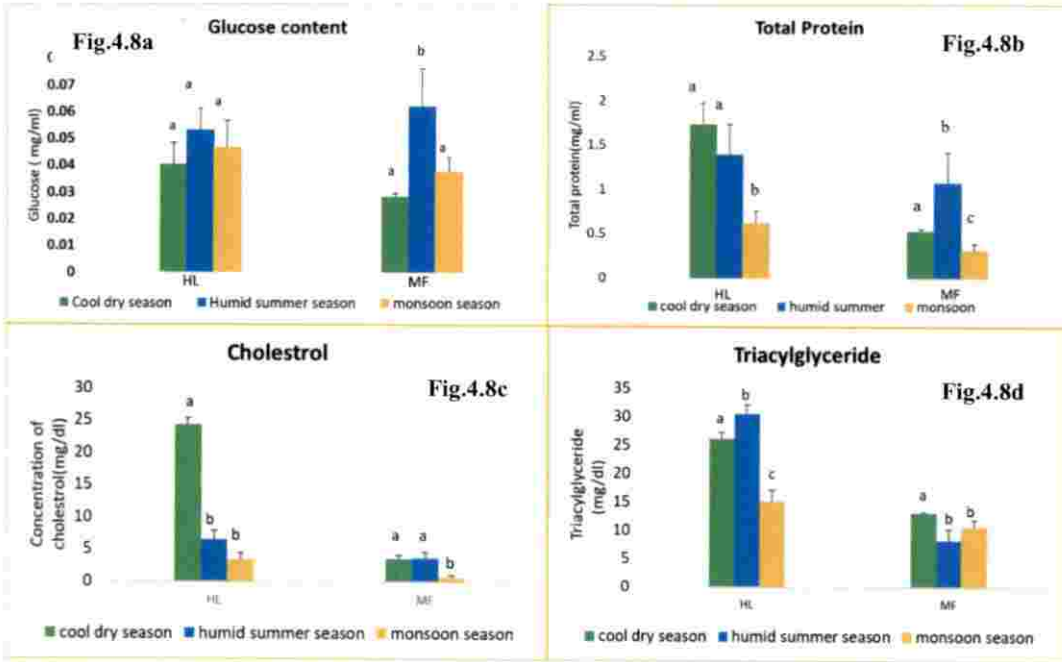

Fig. 4.8 Clustered bar diagram showing levels of biochemical attributes in haemolymph and mantle fluid

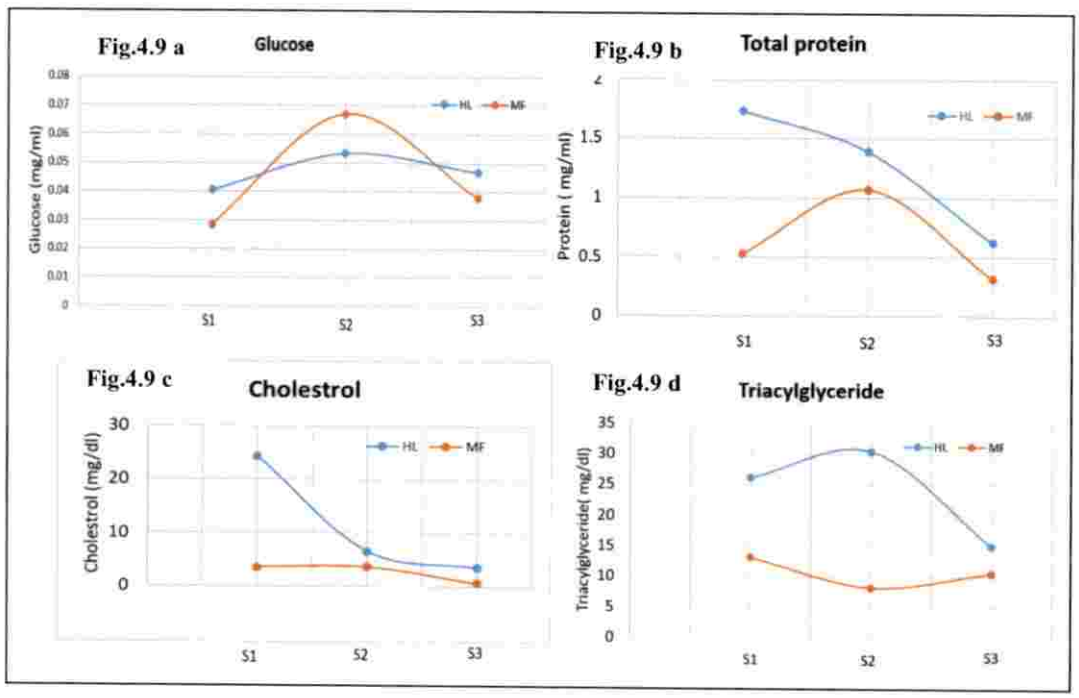

Fig. 4.9 Trend line graph showing levels of biochemical attributes in haemolymph and mantle fluid

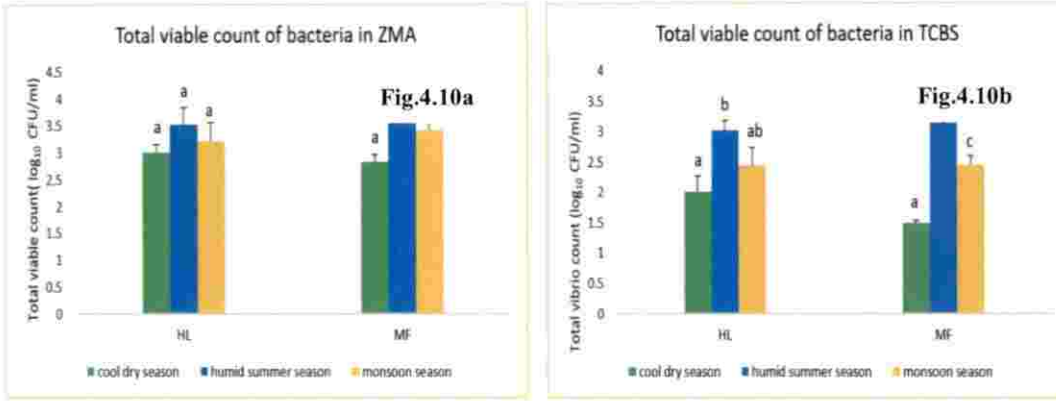

Fig. 4.10 Clustered bar diagram showing Total viable count of bacteria in haemolymph and mantle fluid

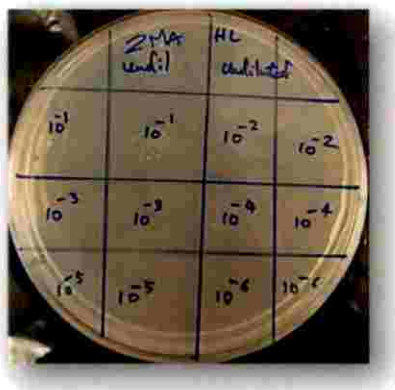

Plate 4.4 ZMA plate

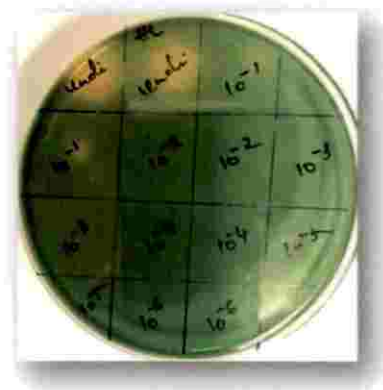

Plate 4.5 TCBS plate

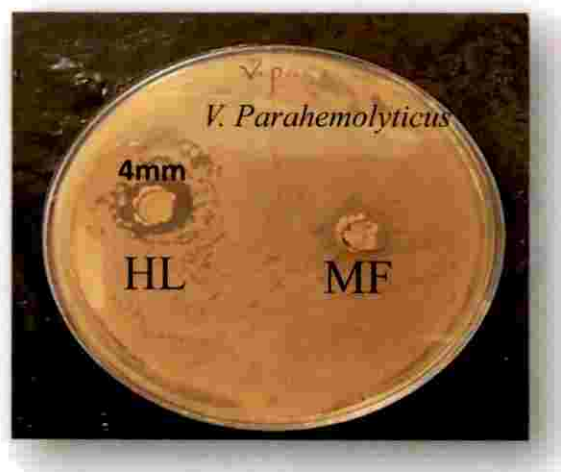

Plate 4.6 Antibacterial activity showed by haemolymph against *V. parahemolyticus* on MHA plates

4.5 SEASONAL CROSS CORRELATION ANALYSIS OF IMMUNO-BIOCHEMICAL ATTRIBUTES

4.5.1 Seasonal cross correlation analysis of immune-biochemical attributes of haemolymph

It was observed that there is no consistency correlation between any of the parameters studied when comparing all the three seasons studied. Individually in each seasons some parameters showed positive as well negative correlation with other parameters. But no parameters showed consistency in correlation circanually. In cool dry season glucose showed positive correlation with condition index. But no significant correlation was observed between glucose and any attributes attributes in other two seasons. Superoxide anion generation is showing a negative correlation with total hemocyte count. However, total hemocyte and cholesterol maintained a positive correlation (Fig. 4.11a). Unlike cool dry season, parameters in humid summer season did not showed any significant correlation with each other (Fig. 4.11b). In monsoon season no significant correlation was observed except a positive between phenoloxidase activity and cholesterol (Fig.4.11c).

4.5.2 Seasonal cross correlation analysis of immune-biochemical attributes of mantle fluid

Seasonal cross correlation of mantle fluid is entirely different from that of haemolymph. Like haemolymph no consistent significant correlation was observed between any of the attributes in three seasons. In cool dry season glucose showed a negative correlation with protein and a positive correlation with cholesterol and cholesterol also showed a negative correlation with protein. Another significant correlation was observed between triacylglyceride, phenoloxidase activity and condition index. Triacylglyceride showed a negative correlation with both condition index and phenoloxidase activity while phenoloxidase activity and condition index are positively correlated (Fig. 4.12a). In humid summer season none of the

parameters showed significant correlation expect a negative correlation between superoxide anion generation and condition index (Fig. 4.12b). Surprisingly, no significant correlation was observed in third season (Fig.4.12c).

4.5.3 Correlation between haemolymph and mantle fluid of *P. viridis*

No consistent pattern was observed in any of the parameters in haemolymph as well as mantle fluid in all the three seasons. In cool dry season and humid summer season protein level in haemolymph and mantle fluid maintained a positive correlation with a varied R value. In cool dry season glucose level in haemolymph showed a positive correlation with phenoloxidase activity of mantle fluid and it showed negative correlation with triacylglyceride of mantle fluid. Superoxide anion generation of haemolymph was negatively correlated with superoxide anion production in mantle fluid, while superoxide anion generation of mantle fluid was negatively correlated with cholesterol level in mantle fluid. Apart from the positive correlation in protein levels no parameters showed same patterns in humid summer season. A negative correlation between cholesterol level in mantle fluid and triacylglyceride level in haemolymph was observed. In contrast to the humid summer season, protein level of mantle fluid in monsoon season showed a negative correlation with phenoloxidase activity and cholesterol level of haemolymph; whereas the cholesterol level of haemolymph was positively correlated with phenoloxidase activity of mantle fluid.

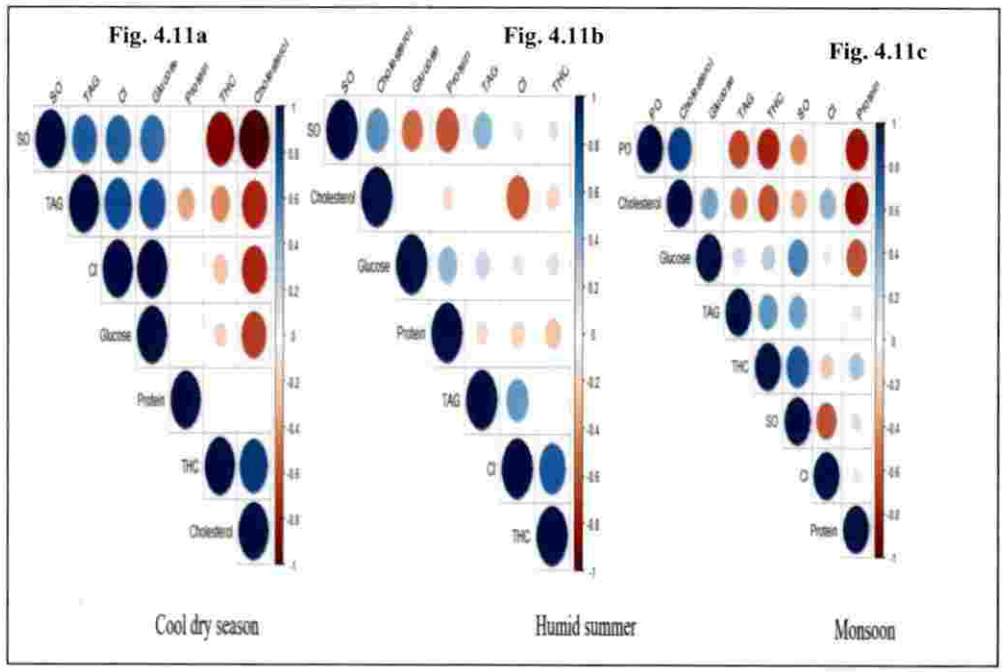

Fig 4.11 Picture showing correlation between various immuno biochemical parameters in haemolymph during three seasons

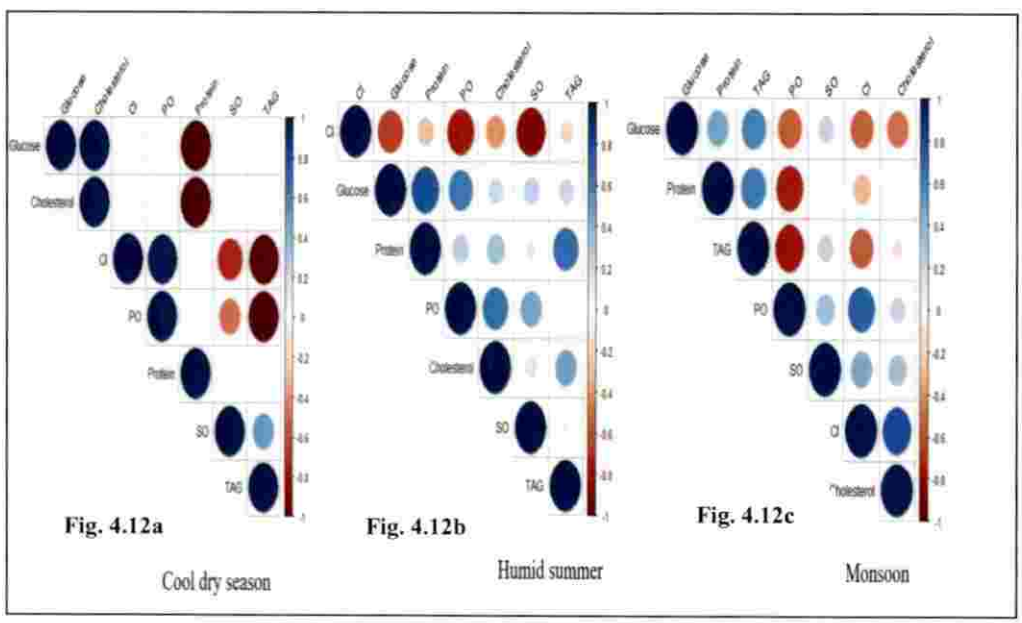

Fig 4.12 Picture showing correlation between various immuno biochemical parameters in mantle fluid during three seasons

4.6 MOLECULAR CHARACTERISATION OF *FERRITIN* GENE IN *P. viridis*

4.6.1 Partial characterization of *ferritin* gene

Total RNA was initially extracted from the mantle tissue of *P. viridis*. The concentration of total RNA was found to be 398 ng/µl. Then using 1 µg of this RNA, cDNA was synthesized using random hexamer primers. Then the *ferritin* gene was amplified using degenerate primers that correspond to the region which belongs to the conserved metal binding sites of the ferritin family members as described previously (Zhang *et al.*, 2003). The optimum temperature for this study was found to be 51^0C and the exact size of the partial gene was determined to be 354 bp in length (Plate 4.7). Then the *ferritin* gene fragment was amplified using the optimized conditions (Table 4.4) which were then sent for sequencing. The obtained sequence after sequencing, BLAST search was followed which revealed one kind of novel ferritin cDNA fragment.

Table 4.4 Optimized PCR conditions for the amplification of *ferritin* gene

Reaction component	Final concentration
Nuclease free water	19.75 µl
PCR buffer	1 X
dNTPs	200 µM of each dNTP
Forward primer	1 µM
Reverse primer	1 µM
Taq enzyme	1 U
cDNA	2 µg
Total Volume	25 µl

4.6.2 3' RACE of *ferritin* gene:

Total RNA was initially extracted from the mantle tissue of *P. viridis*. The concentration of total RNA was found to be 512 ng/µl. Then using 1 µg of this RNA, cDNA was synthesized using oligodT primer. Then the *ferritin* gene was amplified using a specific primer Fer 3 For and PCR anchor primer (Table 3.2). The optimum temperature for the amplification of 3' end of *ferritin* gene was found to be 55^0C and the size of the partial gene was determined to be around 500 bp in length. Gel picture of 3' end of *ferritin* after Agarose gel electrophoresis is shown in figure (Plate 4.8). PCR product was purified, ligated in pTZ57R/T vector and transformed in E. coli DH5α cells. Positive clones were selected by blue white screening and confirmed by doing colony PCR using specific primers, Fer 3 For and PCR anchor primer (Table 3.2). Plasmid was then isolated from positive colonies and a PCR was done using M13 forward and reverse primer and amplified the gene of interest flanking M13 primer sequences. PCR product was then given for sequencing using M13 forward primer. The obtained sequence after sequencing BLAST search was followed which showed similarities with ferritin sequences of eukaryotes.

4.6.3 5' RACE of *ferritin* gene

Total RNA was initially extracted from the mantle tissue of *P. viridis*. The concentration of total RNA was found to be 492 ng/µl. Then using 1 µg of this RNA, cDNA was synthesized using a gene specific primer Fer 5'cDNA. (Table 3.2) Then the cDNA was purified and dA tailing was done using terminal transferase. The 5' end of ferritin gene was amplified using a specific primer Fer 5 Rev and dT anchor primer (Table 3.2). The optimum temperature for the amplification of 3' end of *ferritin* gene was found to be 44^0C and the size of the partial gene was determined to be around 300 bp in length. Gel picture of 5' end of *ferritin* after agarose gel electrophoresis is shown in Plate 4.9. PCR product was purified, ligated in pTZ57R/T

vector and transformed in *E. coli* DH5α cells. Positive clones were selected by blue white screening and confirmed by doing colony PCR using specific primers, Fer 5 Rev and dTanchor primer (Table 3.2). Plasmid was then isolated from positive colonies and a PCR was done using M13 forward and reverse primer and amplified the gene of interest flanking M13 primer sequences. PCR product was then given for sequencing using M13 reverse primer. The obtained sequence after sequencing BLAST search was followed which showed similarities with ferritin sequences of eukaryotes.

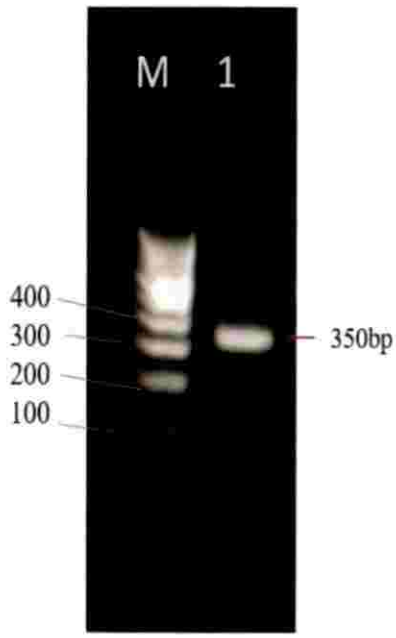

Plate 4.7 Gel picture of PCR product of *ferritin* gene
M- Marker
Lane 1 - PCR product amplified at 51°C

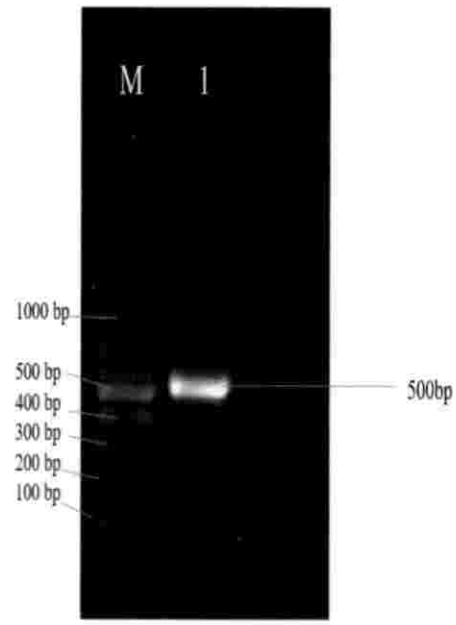

Plate 4.8 Gel picture of PCR product of 3' end of *ferritin* gene
M- Marker
Lane 1 -PCR product

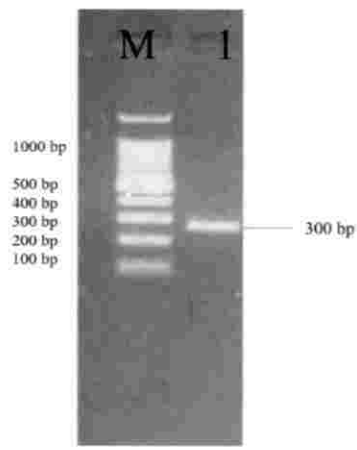

Plate 4.9 Gel picture of PCR product of 5' end of *ferritin* gene
M- Marker
Lane 1 -PCR product

4.6.4 Sequence analysis of *ferritin* gene

Sequences of 3' RACE and 5' RACE were joined using EditseQ and the size of the ferritin gene was determined to be 798 bp in length. Then the sequence was subjected to BLAST search which showed similarities with ferritin sequences of eukaryotes (Table 4.5). Sequence obtained for *ferritin* gene of *P. viridis* was then submitted to GenBank and got an accession number: MN418226. The nucleotide sequence is converted to amino acid sequence then compared with those in GenBank database, NCBI using BLASTP with default parameters.

Table 4.5 NCBI-BLAST search results of *P. viridis ferritin* gene sequence

Organism	Accession number	Query cover %	Identity %
Mytilus edulis	HQ690243.1	61	86.30
Mytilus galloprovincialis	FJ621491.1	56	85.56
Mytilus chilensis	HQ693568.1	53	86.74
Agropecten irradians	HQ225741.1	73	76.37
Pinctada maxima	GQ139542.1	61	77.37
Haliotis discus hannai	KT943445.1	62	75.84
Haliotis diversicolor	FJ594997.1	62	75.89
Biomphalaria glabrata	XM_013225380.1	54	76.19

Organism	Accession number	Query cover%	Identity %
Haliotis rufescens	MH006612.1	62	74.85
Ruditapes decussatus	JX426098.1	53	76.18
Meretrix meretrix	DQ069277.1	74	73.95
Solan grandis	JX462607.2	52	75.06

4.6.5. Phylogenetic analysis of *P. viridis ferritin* gene sequence

The sequence obtained for *ferritin* gene of *P. viridis* was then aligned with the published *ferritin* gene sequences of various organisms. A total of 36 ferritin sequences of various organisms were chosen for homology checking (Plate 4.10). The homology in *ferritin* gene of these 35 different organisms were 21.2%-96.9% at nucleotide level. The highest homology of 96.9% was observed between *Haliotis discus discus and Haliotis diversicolor* which might be due to the fact that both of them belonged to same genus. The highest homology of *ferritin* gene of *P. viridis* was found with *Mytilus edulis* (84.6%) (Plate 4.10). The sequence obtained for *ferritin* gene of *P. viridis* was then aligned with the published *ferritin* gene sequences of various organisms. The aligned sequences were given as input for phylogenetic analysis and the resulted phylogram demonstrating the evolutionary relationships of the 35 *ferritin* sequences were represented in the Plate 4.11. There are two major lineages, A and B corresponding to the two separate sets of *ferritin* gene types one belonged to *P. mirabilis*, a prokaryotic organism and the other set represented by gene sequence of eukaryotes.

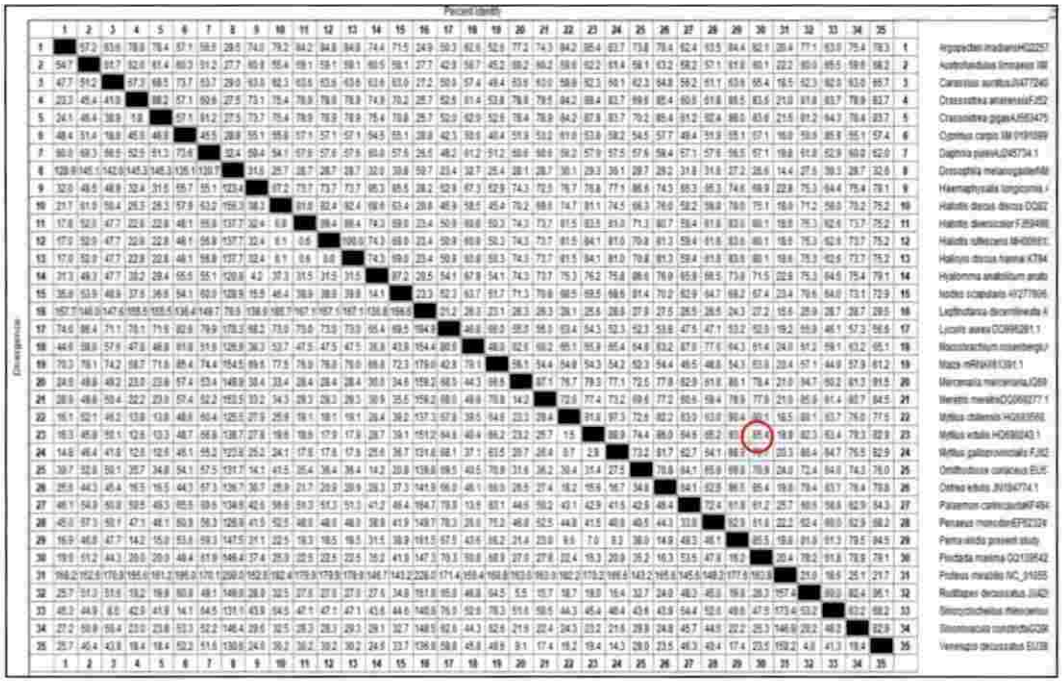

Plate 4.10 Picture showing sequence distance of various *ferritin* gene sequences

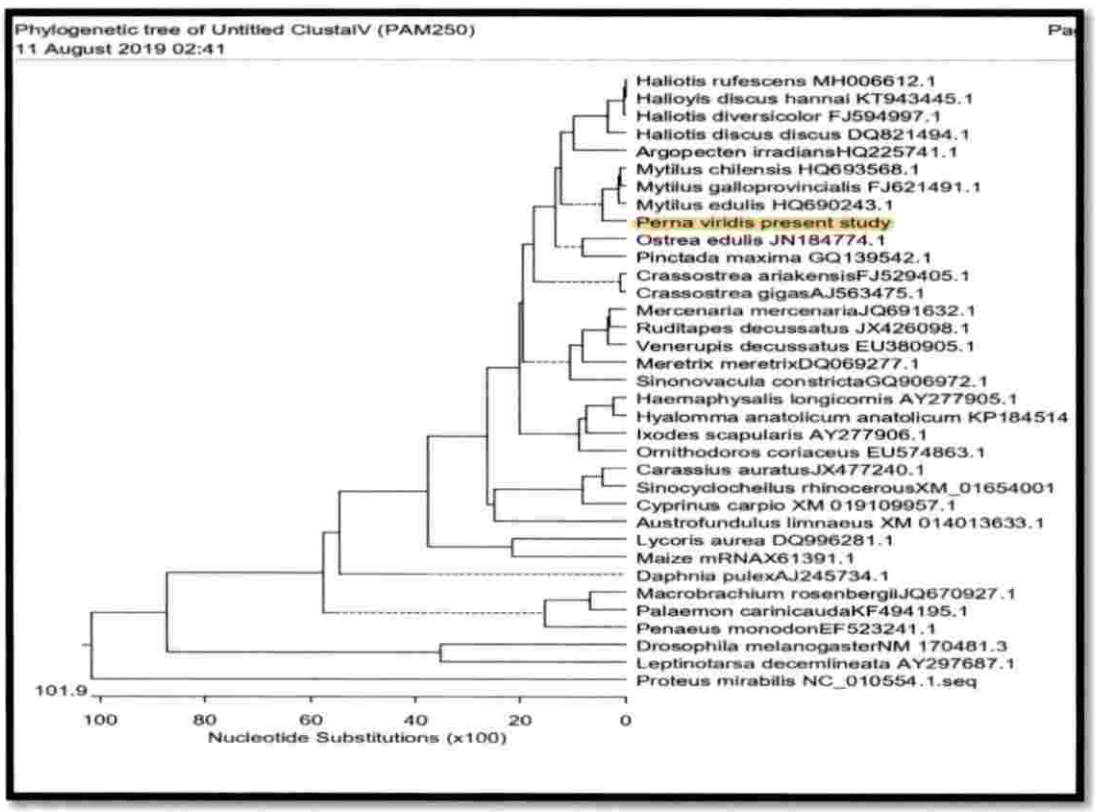

Plate 4.11 Phylogenetic relationships of the *ferritin* gene of *P. viridis* with other organisms

4.6.6. Features of the predicted ferritin protein

The sequence was then analyzed through ORF Finder to find the open reading frame (ORF) region which revealed that the longest open reading frame was of 522 bp. The deduced ferritin protein consisted of 173 amino acid residues with a calculated molecular mass of 19.9 kDa. The sequence also contained 112 bp as 5' untranslated region (UTR) and a poly(A) tail at its 3' end. The amino acid sequence of the deduced ferritin protein was then compared in GenBank database, NCBI using BLASTP with default parameters (Table 4.6).

Table 4.6 NCBI-BLAST search results of *P. viridis* ferritin protein sequence

Organism	Accession number	Cover %	Identity %
Mytilus edulis	AEM36072.1	94	93.29
Mytilus chilensis	AEE60904.1	82	92.31
Crassostrea virginica	XP_022294039.1	97	91.07
Mytilus galloprovincialis	ACM86786.1	86	90.67
Pinctada fuctada	AAQ12076.1	95	89.16
Crassostrea gigas	XP_011432947.1	97	88.69
Crassostrea ariakensis	ACU25551.1	96	88.62
Ostrea edulis	AFK737038.1	97	88.10
Agropecten irradians	ADR71732.1	98	85.38
Haliotis diversicolor	ABY87353.1	98	85.29
Agropecten purpuratus	ALV83427.1	98	84.21
Haliotis rufescens	ACZ73270.1	98	84.12
Pectinaria gouldii	ACJ37.69.1	98	83.63
Haliotis discus hanna	ABH10672.1	98	82.94
Mizhuopecten yessoensis	XP_021355362.1	99	82.56
Ruditapes decussatus	AFX93744.1	97	82.74
Mereterix meretrix	AAZ20754.1	97	80.36

Overall, the homology in ferritin protein among these 35 different organisms were 20.4%- 99.4% at AA level (Plate 4.12). The highest homology of 99.4% was

observed between *Haliotis rufescens and Haliotis diversicolor* which might be due to the fact that both of them belonged to same genus. The highest homology of ferritin protein of *P. viridis* was found with *Mytilus edulis* (93.3%) (Plate 4.12). However, 3' untranslated region (UTR) and 5' UTR region could not be compared as complete sequence of *ferritin* gene of these organism was not available.

The conserved domain search from NCBI identified that the sequence has Euk_Ferritin domain- at 11 aa - 168 aa levels (domain architecture ID 10099405 and Accession cd01056) which belongs to Ferritin-like superfamily of diiron-containing four-helix-bundle proteins. The domain contained 3 structurally conserved features namely, ferroxidase diiron center, ferrihydrite nucleation center and iron ion channel. The protein structure was predicted by RaptorX structure prediction server (Plate 4.13).

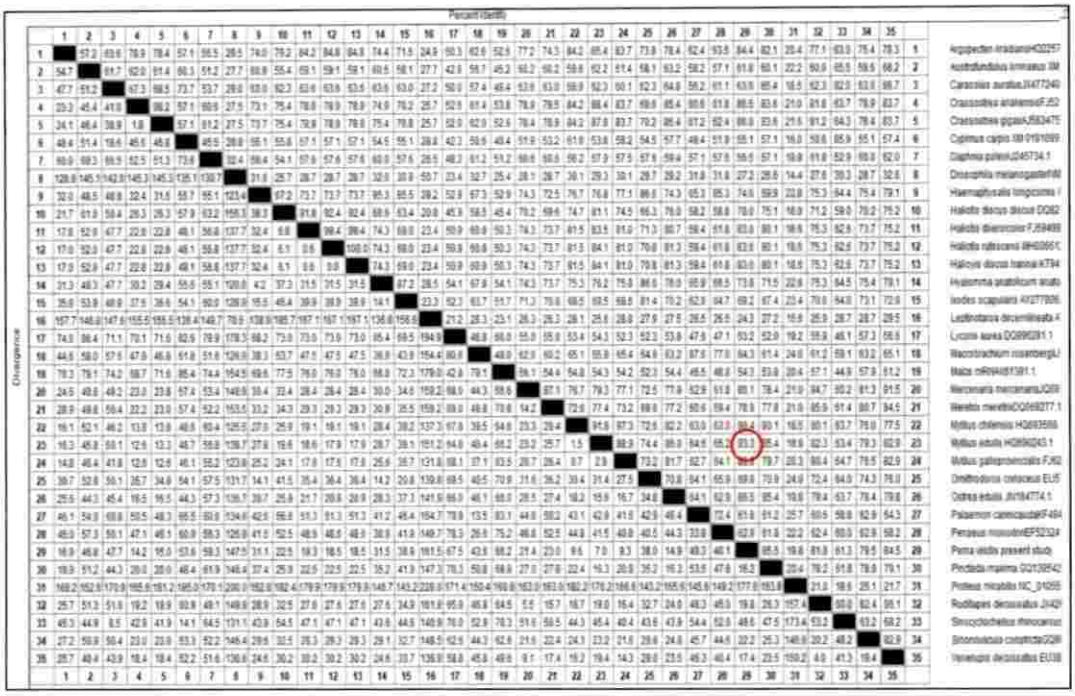

Plate 4.12 Picture showing sequence distance of various ferritin amino acid sequences

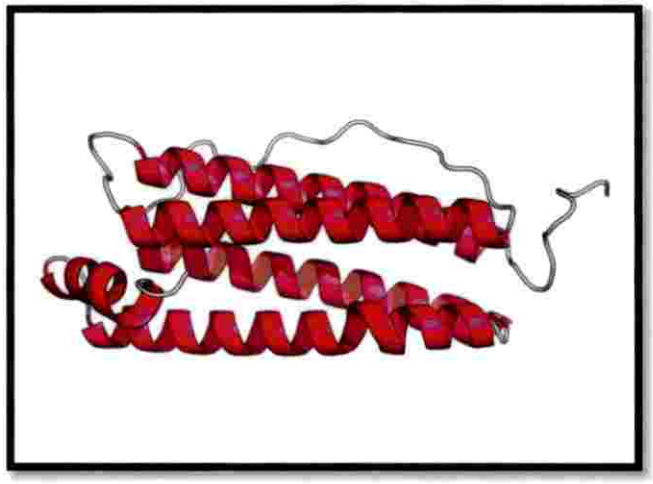

Plate 4.13 Picture showing predicted structure of ferritin protein in *P. viridis*

Physicochemical properties of the protein were then predicted using ProtParam Predicted isoelectric point (pI) and molecular weight for the *ferritin* gene was 4.97 and 19970.24 respectively. Total number of negatively charged residues (Asp + Glu) was predicted as 31; Total number of positively charged residues (Arg + Lys) as 21. The predicted amino acid composition of Ferritin protein in *P. viridis* was given in the Table 4.7.

Table 4.7 Predicted amino acid composition of ferritin protein in *P. viridis*

Name of amino acid	Code	Numbers	Composition %
Alanine	A	12	6.9
Arginine	R	9	5.2
Asparagine	N	7	4
Aspartic acid	D	14	8.1
Cysteine	C	2	1.2
Glutamine	Q	12	6.9
Glycine	G	9	5.2
Histidine	H	5	2.9
Isoleucine	I	11	6.4
Leucine	L	13	7.5
Lysine	K	12	6.9
Methionine	M	6	3.5
Phenylalanine	F	7	4.0
Proline	P	3	1.7
Serine	S	16	9.2
Threonine	T	3	1.7
Tryptophan	W	1	0.6
Tyrosine	Y	8	4.6
Valine	V	6	3.5

Pyrolysine	O	0	0.0
Selenocysteine	U	0	0.0

The atomic composition of the predicted protein was given in Table 4.8.

Table 4.8 Atomic composition of the predicted protein

Carbon C	870
Hydrogen H	1352
Nitrogen N	242
Oxygen O	282
Total number of atoms	**2754**

The other features of the predicted protein were tabulated in Table 4.9.

Table 4.9 Features of the predicted protein

Formula	$C_{870}H_{1352}N_{242}O_{282}S_8$
Extinction efficient, $M^{-1}cm$-1 (assuming all pairs of Cys residues from cystines)	17545
Extinction efficient, $M^{-1}cm$-1 (assuming all pairs of Cys residues are reduced)	17420
Instability index (instability index 2)	52.35
Aliphatic index	71.10
Grand average of hydropathicity	-0.758
N-terminal of the sequence	Met, M
Estimated half-life	30 h in mammalian reticulocytes, *in vitro;* 20 h in yeast *in-vivo;* 10 h in *E. coli*, *in-vivo*

DISCUSSION

5. DISCUSSION

The present study provides seasonal baseline data of 11 relevant haemolymph parameters and mantle fluid parameters for Asian green mussel, *Perna viridis* to predict the eco-immunological status of apparently healthy, *P. viridis*. The entire sampling was done three times a year namely, cool dry season (November to February), humid summer season (March to May) and monsoon season (June to October) which was categorized based on average temperature and average precipitation coinciding the spawning season (May- June) for *P. viridis* in Indian coast (IMD, 2018). Overall, 36 animals were pooled to 6 groups and haemolymph and mantle fluid were collected in each season for different analysis. Total viable count, Total *Vibrio* count and antibacterial activity is screened for the haemolymph as well as mantle fluid for each season. Subsidiary to this, relevant immune-biochemical parameters for the haemolymph such as Total hemocyte count, superoxide anion generation, phenol oxidase activity, lysozyme activity, glucose content, total protein, albumin, cholesterol, triacylglyceride were also evaluated for each samples. Further, mantle fluid was also evaluated for superoxide anion generation, phenol oxidase activity, lysozyme activity, glucose content, total protein, albumin, cholesterol, triacylglyceride in each season. The prime objective of the study was to understand the seasonal impacts on immuno-biochemical attributes in the haemolymph and mantle fluid and their contribution towards modulating ecophysiology. In order to minimize the physiological variations due to anthropogenic contaminants, the sampling site with minimal anthropogenic interventions like urban and industrial pollutions was selected for the present study. To evaluate the effect of different environment parameters on the innate immune/biochemical parameters, different environmental parameters such as temperature, salinity, pH, and dissolved oxygen corresponding to sampling site and season were noted each time along with the sampling.

As per previous literatures, haemocytes and soluble haemolymph factors contributed effectively in modulating innate immune systems of invertebrates (Galloway and Depledge, 2001; Canesi et al., 2006; Galloway and Goven, 2006). Similar way, a few studies dealing with oyster pallial cavity fluid have demonstrated the presence of different immune molecules in the mantle fluid (Mc Dade and Tripp 1967). Moreover, as per Allam and Paillard, (1998), the haemolymph as well as mantle fluid plays a comprehensive role in defining the bivalve defence strategy, and according to Matozzo *et al.* (2003), functional response of mussel's immune system appears closely dependent on seasonal variations in terms of environmental parameters and the physiological status of clams. So, seasonal aspects of viable counts, antibacterial activity and immuno-biochemical attributes of both haemolymph and mantle fluid is used in the present study to elucidate the comprehensive ecophysiology and health status of *P. viridis,* which will be helpful to predict various stressful conditions in mussel culture as well as in the environmental monitoring studies of various pollutants.

Condition index showed an increasing trend in the first two seasons indicating somatic growth, and from summer to monsoon season (spawning period) condition index was appeared to be comparable. However, the inter-relations of somatic growth and reproduction in mussels varied between tropical and temperate regions. Studies showed that gametogenesis proceeds at the expense of somatic growth in the tropical *Moira atrapus* (Moore and Lopez, 1966) and somatic and gonadal growth appear closely coupled in temperate *Ostrea edulis* beyond the minimal threshold for gametogenesis (Mann, 1979).

The haemolymph cells or haemocytes play a key role in the implementation of a non-specific immune system of mussels and vary significantly with the physiological state of the animals and are determined by many environmental factors (Anisimova *et al.,* 2017). Studies reported that elevation of the total hemocyte count is an indication for the augmentation of immunity of invertebrates (Kacsoh and

schlenke, 2012) and acts as a marker for environmental stress (Mello *et al.,* 2010). Yet, there is no significant difference observed in THC between three seasons for the present investigation for apparently healthy mussels. Reports suggested that data associated with THC in bivalves are under dispute (Matozzo and Marin, 2011; Perringauld *et al.,* 2011) as correlation between THC and environmental observed for many bivalves (Paillard *et al.,* 2004). Temperature is one of the important factor linked to THC of filter feeding animals like mussels and correlation between temperature and THC vary between species and other ambient conditions (Fischer and Oliver, 1996; Carballal *et al.,* 1998;). In accordance to observations of Fischer and Oliver, (1996), the study showed a lower average THC level in summer.

Phenoloxidase cascade system is considered as an effector mechanism in invertebrates from decades (Smith *et al.,* 1991; Hemandez-lopez *et al.,* 1996). Activation of phenoloxidase enzyme in molluscs from the inactive form ProPO when stimulated by any external injury confirming its role in defense mechanism (Peters and Raftos, 2003; Esteban *et al.,* 2006; Aladaileh *et al.,* 2007, Yu *et al.,* 2011). So, the impact of seasons on phenoloxidase activity of haemolymph and mantle fluid was evaluated to evaluate its seasonal effects. However, phenoloxidase activity in haemolymph and mantle fluid differed significantly in all the three seasons for the present study. More importantly, higher PO activity was observed in monsoon season (spawning period), contradicting to the observations of Yu *et al.* (2011), where he observed a decrease in PO activity in the spawned clams, *Mactra veneriformis*. Further investigations are required in this scenario due to lack of similar literatures to compare. The environmental stress and suppression of immune responses has been defined in a range of invertebrate models and is well studied in different molluscs too. The lowest PO activity observed in summer for the present study may be attributed to the increased environmental stress due to rise in temperature (Traves *et al.,* 2008; Kuchel *et al.,* 2012). However, researchers suggest an alternate role for PO in mantle, the PO in mantle fluid aids in the formation of periostraca which is

composed of quinone-tanned protein (Misogianes, 1979) where quinone, the crosslinking agent is considered fundamental to the inertness and durability of mantle layer. Since this layer is formed before all other portions of the exoskeleton, it must polymerize rapidly to resist solubilization (Waite and Wilber, 1976). So an increasing trend for mantle PO is observed seasonally in the present study could be linked to the increasing condition index from cool dry season to monsoon season followed by thick shell formation during the spawning period (monsoon season).

When haemolymph is stimulated by any external factor, monovalent and toxic reactive oxygen species like superoxide, hydoxy radicals and hydrogen peroxides are produced and it involves in cell disruption and killing. This cellular defense reaction is recognized as an immunologically important one and identified in almost all molluscs (Adema *et al.* 1991; Arumugam *et al.* 2000). So we evaluated the generation of superoxide anions in haemolymph and mantle fluid in apparently healthy mussel and haemolymph superoxide generation showed a significant difference across seasons. Lowest value of haemolymph superoxide generation is observed in monsoon season, where salinity was apparently lower. The experimental condition of hypo salinity and hypoxia already showed a lower ROS production in the hemocyte of *P. viridis* (Lushchak, 2011). More importantly, lowest superoxide anion generation was observed in summer season for the present study, where reports already suggested a lower granulocyte density and their immune functions of *Crassostrea virginica* and *Mercenana mercenaria*n in summer seasons (Foley and Cheng, 1975). Mantle hemocytes are functionally active as demonstrated by their ability to have different immune functions and usually actively transported via trans-epithelial migration from the hemocoel (Allam, 1998; Allam and Paillard, 1998). The contribution of such peripheral hemocytes towards superoxide anion production is under-investigated in apparently healthy molluscs, however these cells are assigned to have a sentinel function (Allam and Pales Espinosa, 2016). Nevertheless, present study showed a significant variation in mantle fluid superoxide anion generation.

Glucose is the principal monosaccharide present in the haemolymph of most invertebrates and stable glucose haemolymph levels are very important for the regular functioning of the nervous, muscle and reproductive systems. Further, glucose can be accumulated in the form of glycogen in many invertebrates (Vinagre and Da silva, 1992,) yet their storage location varies from species to species (Johnston and Davies, 1972). Studies carried out in molluscs also showed haemolymph glucose as the integral sugar for these animals. (Goudsmit, 1972). Further, literature survey indicated a significant variation in haemolymph glucose levels of mussels, post infection and increased circulating glucose in response to season, weather extremes, handling stress or any recent environmental stress require extra fuel in mussels (Pekkarinen and Suoranta 1995; Gustafson *et al.,* 2005; Holopainen 1987; Pekkarinen 1997). Contrasting to their observations, no significant difference was observed in haemolymph glucose levels between three seasons for the present study; while glucose level in mantle fluid showed a significant difference. Unlikely, reports suggested a direct linkage of tissue glucose level to growth and gametogenesis of mussels (Quayle, 1943; Loosanoff, 1965). Supporting this, later investigations confirmed that mussels store energy as glycogen, which accumulates in the mantle tissue and is subsequently used during gametogenesis (Bayne *et al.* 1982). Further, a concurrent cycles of glycogen storage and gametogenesis in mantle tissue were reported in many bivalves (Honkoop 2003, Ren *et al.* 2003; Farias *et al.* 1997; Darriba *et al.* 2005), substantiating a higher glucose level following a drop from summer to monsoon season observed for both haemolymph and mantle fluid in the present study. However, Pekkarinen, (1997) observed that glucose may supply energy for the outer mantle epithelium and it could be a precursor for synthesis of the conchiolin matrix of the shell. Thus, the boost of mantle fluid glucose level from cool dry season to humid summer season can be attributed to expenditure for increasing the shell size in present study. Nevertheless, as per Velez and Epifanio (1981), seasonal pattern for haemolymph glucose vary between species. Further, many studies indicate a significant variation in haemolymph glucose levels of mussels, post

infection. The least variability observed in haemolymph glucose level between the seasons in the present study could be indicating their apparently balanced physiological status, yet the effect on growth and gametogenesis on haemolymph glucose for *P. viridis* has to be investigated as no similar literature is available for further comparison. However, our results indicated that average value of haemolymph glucose peak in summer season which is in agreement with the studies by Pekkarinen, (1997).

Haemolymph protein levels fluctuate with changes in environmental and physiological conditions and play crucial roles in the physiology, reproduction and stress responses of many invertebrates (Lin and Chen, 2001). Further, Feng and Canzonier, (1970); Ford, (1986); Chu and La Peyre, (1993); Cheng (1969), reported a natural decrement of protein level in haemolymph of infected animals. The present study showed a contradictory pattern between haemolymph protein and haemolymph glucose levels in cool dry season and humid summer season. The possible reason for lower values of haemolymph protein in humid summer season may be a metabolic shift away from carbohydrates in glycogen-depleted conditions (Gustafson *et al.*, 2005). Further, the level of haemolymph protein was found to be least in spawning season (Monsoon season) in the present study. This may be due to the reproduction-immunity trade-offs reported in molluscs (Cho and Jeong, 2005; Li *et al.*, 2007; Samain *et al.*, 2007; Wendling and Wegner, 2013) where physiological costs of reproduction frequently involve the decrease in both basal and induced levels of immunity and reallocation of a common energy source like protein and glucose (Schwenke *et al.*, 2016). Present study showed a significance difference in mantle fluid protein level in all three seasons, and highest value is observed in summer season. The possible reason for the increase of mantle fluid protein level during summer season may be the active secretion of proteins into the extra pallial space for aiding the shell formation during maturation (Young *et al.*,1977).

Different forms of lipid like cholesterol and triacylglyceride are essential for the growth, and reproduction of invertebrates (Vikas *et al.*, 2018). Though cholesterol is the major sterol in invertebrates (Vikas *et al.*, 2018), juvenile have lower body lipid levels than adult animals and they accumulate body lipids over the life in the form of triacylglycerol (Correia *et al.*, 2003) and provide main source for reproductive energy (Wouters *et al.*, 2001). However, cholesterol is essential component in the cell membranes structure and for their gonad development and reproductive cycle (Wouters *et al.*, 2001). The present study also indicated a gradual decrease in cholesterol level with corresponding increase in somatic growth (from cool dry to humid summer season). The lower level of cholesterol observed in monsoon season could be their active investment for gonad development during spawning (Castiglioni *et al.*, 2009). Precisely, average triglyceride level was higher in the summer season in the present study and Previous reports in scallops have showed that adult scallops are shown to synthesize lipids *''de novo''* for gametogenesis, using stored energy reserves such as triglycerides (Vassalo, 1973; Barber and Blake, 1985; Napolitano and Ackman, 1993). This may attribute to sudden rise in haemolymph triacylgyceride level in summer season prior to spawning season. The level of lipid was lower in spawning season for the present study and reports already indicated that energetic lipid reserves were being transferred gonads via the haemolymph during gametogenesis in spawning season (Napolitano & Ackman 1993, Soudant *et al.* 1996, Caers *et al.* 1999, 2003, Birkely *et al.* 2003, Martínez-Pita *et al.* 2012b). Gametogenesis and energy storage occur in the mantle tissue indicating a higher level of lipid (Cholesterol and triglycerides) level in the mantle fluid during the monsoon season. Further investigations on lipid profiling of mantle tissue is needed to enlight more on the seasonal dynamics of lipid mobilization between haemolymph and mantle tissue; especially at the time of gametogenesis.

A reproduction-immunity trade-off is observed for protein, cholesterol and triglyceride in hemolymph with lower value observed for spawning season. However, none of the parameters studied showed a consistent significant correlation

pattern ($P<0.001$) with respect to season. Further investigations are required to pave light on the seasonal dynamics of metabolic interaction and their immune outcome through interlinking of these attributes to tissues and body fluids with their active transport in *P. viridis*.

The *ferritin* gene, one of the immune genes was successfully isolated and fully characterized in *P. viridis* for the first time. The full length of *ferritin* cDNA was determined to be 798 bp containing 5' untranslated region, a 3' poly A tail and a deduced amino acid of 173. The sequence was submitted to GenBank (Sequence ID:2260881) and got an accession number 'MN418226'. Both nucleotide and amino acid sequence of *ferritin* obtained in our study showed highest similarity with *Mytilus edulis* (84.9% and 93.3%). This might be due to the fact that both of them belongs to the same family Mytilidae. Ferritin is recognized an ant oxidative protein that inhibits Reactive Oxygen Species(ROS) production through the Fenton reaction (Wang *et al.*,2009). The three structurally conserved features -ferroxidase diiron center, ferrihydrite nucleation center and iron ion channel present in ferritin protein obtained in our study showed that this mussel ferritin has ferroxidase ability. Ferroxidation is the main step in iron storage and inhibition of formation of ROS (Orino *et al.*, 2001). Thus the results of the study can serve as a reference for further investigation on the disease management studies on *P. viridis* as well as on the marine pollution monitoring studies using the same mussel.

SUMMARY

6. SUMMARY

Perna viridis (Asian green mussel), is a valuable food source for human consumption and it is one of the candidate species for aquaculture. Due to their specific ecology, and feeding habit, these species are further utilized in biomonitoring programmes also. However, further research is required to improve the available technology and to increase production for these filter feeder organisms. Information regarding various stress related parameters of apparently healthy *P. viridis* is very crucial in the investigation and management of various diseases as well as in pollution monitoring studies, which is lacking currently. Moreover, to achieve this, base line information on the various stress parameters as well as their seasonal modulations for healthy organisms are necessary. The objective of the study was to understand the seasonal impacts on immuno-biochemical attributes in the haemolymph and mantle fluid and their contribution towards modulating ecophysiology. As a holistic approach towards eco-immunology, the present study also aimed to isolate and characterize the *ferritin* gene of *P. viridis*, an important protein which has role in the incorporation of iron into the shell of bivalves and in immunity protections against various diseases.

Circannually, three seasons namely cool dry season (November to February), humid summer season (March to May) and monsoon season (June to October) were selected for the present study. Overall, 36 animals were pooled to 6 groups and haemolymph and mantle fluid of each animal were collected in each season for evaluating various important attributes such as total viable count, total *Vibrio* count, antibacterial activity, total hemocyte count, superoxide anion generation, phenoloxidase activity, lysozyme activity, glucose, total protein, albumin, cholesterol and triacylglyceride content. Mantle tissue was collected for the characterization of *ferritin* gene using RACE PCR. Average condition index of mussels in all three seasons are obtained as 0.97709±0.13 in seasons cool dry season (November-February), 5.5477±0.93 in humid summer season (March-May) and 8.321±0.25 in

monsoon season (June-September). Total hemocyte count of *Perna viridis* was comparable between the three seasons studied, having no significant difference between the seasons ($p>0.05$). The circannual phenoloxidase activity in haemolymph showed a gradual decrement from cool dry season to humid summer seasons following a steep increment towards the monsoon season, while a gradual increment from cool dry season to monsoon season was observed in mantle fluid. A significant difference was observed in superoxide anion production of haemolymph collected in all three seasons ($p=0.034$) with a gradual decrement from cool dry season to humid summer and toward monsoon season also. But in mantle fluid an increase was observed in monsoon season. No significant difference ($p>0.05$) was observed between glucose content in all three seasons with a comparable glucose level. However, in mantle fluid seasonal trend for glucose showed a sharp increase in humid summer followed by a decrease in monsoon season. The average protein value was higher (1.73mg/ml) in cool dry season and least (0.623mg/ml) during monsoon season. Circannual trend for haemolymph protein showed a gradual decrease from cool dry season to humid summer season followed by a sharp decrease in monsoon season. In contrast to haemolymph the protein level in mantle fluid showed an increment from cool dry season to humid summer season. The albumin content of both haemolymph and mantle fluid was observed below the detectable level. Till now no studies have identified the presence of albumin in mussels. Circannual trend for haemolymph cholesterol showed a steep decrease from cool dry season to humid summer season followed by a gradual decrease in monsoon season. Cholesterol content of haemolymph in humid summer season showed a positive correlation with total hemocyte count. Triacylglyceride of haemolymph showed individual significant difference in between each seasons with highest triacylglyceride level in humid summer season (30.47mg/dl) and least value in monsoon season (15.13mg/dl). Unlike haemolymph, cholesterol content in mantle fluid followed a sharp decrease towards monsoon. Circannual pattern of mantle fluid triacylglyceride showed a gradual decrease from cool dry season to humid summer season and a gradual increase in

monsoon season. Both haemolymph and mantle fluid followed a same pattern in total viable count of bacteria and *Vibrio*, an increase in summer compared to other two seasons. It was observed that there is no consistency in correlation between any of the studied parameters circanually. Only protein level in mantle fluid and haemolymph showed a positive correlation in both cool dry season and humid summer season.

It is important to note that a reproduction-immunity trade-off is observed for many of immune-biochemical attributes. Yet none of the parameters studied in present research showed a consistent significant correlation pattern with respect to season. Further investigations are required to pave light on the seasonal dynamics of metabolic interaction and their immune outcome through interlinking of these attributes to tissues and body fluids with their active transport in *P. viridis*. Importantly, the outcome of the study can be exploited in various pollution monitoring studies and disease management strategies for *P. viridis* culture.

In the present study, the full length cDNA of an immune related gene, *ferritin* gene (798 bp) was isolated and characterized for the first time in *P. viridis*. The sequence was submitted to GenBank (Sequence ID:2260881) and got an accession number 'MN418226' The highest homology of *ferritin* gene of *P. viridis* was found with *Mytilus edulis* (84.6%) which might be due to the same family they belonged to. The sequence was then analyzed through ORF Finder revealed that the longest open reading frame was of 522 bp. The deduced ferritin protein consisted of 173 amino acid residues with a calculated molecular mass of 19.9 kDa. A conserved domain, Euk_Ferritin domain- at 11 aa - 168 aa levels (domain architecture ID 10099405 and Accession cd01056) was identified which belongs to Ferritin-like superfamily of diiron-containing four-helix-bundle proteins. The domain contained 3 structurally conserved features namely, ferroxidase diiron center, ferrihydrite nucleation center and iron ion channel. These features have importance in controlling intracellular iron and detoxification mechanism. The result of the study may serve as a reference for further expression studies of the same gene.

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7. REFERENCES

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APPENDICES

8. APPENDICES

I. REAGENTS USED FOR BACTERIOLOGICAL PROCEDURES

1. Zobell Marine Agar

Ingredients	**grams L^{-1}**
Peptic digest of animal tissue	5.000
Yeast extract	1.000
Ferric citrate	0.100
Sodium chloride	19.45
Magnesium chloride	8.800
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Sodium sulphate	3.240
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15.00
Final pH (at 25°C)	7.6±0.2

2. Brain Heart Infusion Agar:

Ingredients	**grams L^{-1}**
Calf brain (infusion form)	200.00
Beef heart (infusion form)	250.00

Proteose peptone	10.00
Dextrose	2.00
Sodium chloride	5.00
Disodium phosphate	2.50
Agar	15.00
Final pH (at 25°C)	7.4±0.2

3. Muller Hinton agar:

Ingredients	**grams L^{-1}**
Beef infusion from	300.00
Casein acid hydrolysate	17.50
Starch	1.50
Agar	17.00
Final pH (at 25°C)	7.3±0.2

4. **TCBS Agar**:

Ingredients	**grams L^{-1}**
Preteose peptone	10.00
Yeast extract	5.00
Sodium thiosulphate	10.00
Sodium citrate	10.00
Oxgall	10.00
Sucrose	20.00
Sodium chloride	10.00
Ferric citrate	1.00
Bromo thymol blue	0.040
Thymol blue	0.040
Agar	15.00
Final pH (at 25°C)	8.6±0.2

II. REAGENTS FOR EVALUATION OF IMMUNO-BIOCHEMICAL PARAMETERS

1. Alkaline copper sulphate reagent

Ingredients	($g\ L^{-1}$)
2% Sodium carbonate	20
0.1 N Sodium hydroxide	4
0.5 % Copper sulphate	5
1%Sodium potassium tartrate	10

2. 0.1 N NaOH

NaOH	4 $g\ L^{-1}$

3. 2M KOH

KOH	112.2 $g\ L^{-1}$

4. Phosphate buffer (pH -7)

1 M potassium dihydrogen phosphate	61.5 ml
1 M dipotassium hydrogen phosphate	38.5 ml

Diluted to 1 L using triple distilled water.

5. Thiourea (5mM)

Thiourea	0.38g L^{-1}

6. Potassium dichromate- acetic acid solution (1:3)

Potassium dichromate (5%)	5g

7. Phosphate Buffer Saline (PBS, pH 7.4)

Sodium chloride	8 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.20 g

Disodium hydrogen phosphate (Na_2HPO_4)	1.10 g
Potassium chloride	0.2 g
Distilled water	1000 ml

Autoclaved at 121°C, 15 lb pressure for 30 min. Stored at 4°C.

8. Neutral buffered saline(NBF)

Formaldehyde	40 ml
Disodium hydrogen phosphate	6 g
Sodium dihydrogen phosphate	4 g
Distilled water	960 ml

III. REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

1. Tris-acetate- EDTA (TBE) buffer (10X)

Tris base	108 g
Boric acid	55 g
Disodium EDTA	7.44g
Final pH	8.2

Triple distilled water was added to make the final volume up to 1000 ml. A working solution of 1X was used.

2. Ethidium bromide stock solution (10 mg/ml)

Ethidium bromide	100 mg
Distilled water	10 ml

The solution was mixed and stored at 4°C. A concentration of 0.5-1 µg/ml was used in preparing agarose gel.

3. Loading dye (6X)

Bromophenol blue	0.5% (w/v)
Glycerol	30%

Prepare in 10 X TBE. Store at 4°C.

IV. REAGENTS FOR CLONING:

1. LB (Luria-Bertani) broth

Bacto tryptone	10 g
Bacto Yeast extract	5 g
NaCl	10 g
Deionized water	950 ml

Adjust pH to 7.4 with 1N NaOH. Volume made up to 1L and sterilized by autoclaving.

Store at 4°C.

2. LB agar

Add 2.5% agar in LB broth and sterilize by autoclaving

3. Ampicillin(100mg/ml)

Ampicillin powder	100 mg
Sterile distilled water	1 ml

1. X- Gal (20mg/ml)

20 mg X gal is dissolved in 1 ml dimethyl sulfoxide (DMSO)

2. IPTG(24mg/ml)

24 mg IPTG is dissolved in 1 ml sterile distilled water.

V. REAGENTS FOR PLASMID DNA ISOLATION BY ALKALINE LYSIS METHOD

1. Solution I (P1) Resuspension buffer

Tris-Cl (pH 8.0)	50 mM
EDTA (pH 8.0)	10 mM

The P1 solution was autoclaved for 15 min and store at 4°C.

2. Solution II (P2) Lysis buffer

NaOH	0.2 N
SDS	1.0%

The solution was prepared fresh and stored at room temperature.

3. Solution III (P3) Neutralization buffer

5 M Potassium acetate (pH 5.5)	60.0 ml
Glacial acetic acid	11.5 ml
Triple Distilled Water	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate and stored at 4°C.

ABSTRACT

EVALUATION OF IMMUNO- BIOCHEMICAL ATTRIBUTES OF ASIAN GREEN LIPPED MUSSEL (*Perna viridis*) AND CHARACTERIZATION OF SELECTED IMMUNE RELATED GENE

by

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Abstract of the Thesis

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9. ABSTRACT

This study entitled 'Evaluation of immuno- biochemical attributes of Asian green lipped mussel (*Perna viridis*) and characterization of selected immune related gene' mainly aimed to analyze the seasonal impacts on biochemical and immunological parameters in the haemolymph and mantle fluid of *Perna viridis* and to characterize an immune related gene (*ferritin*) in *Perna viridis*. *Perna viridis* (Linnaeus, 1758) is one of the economically important marine bivalves which has also been recognized as an ideal aquatic animal for bio-monitoring.

Being a filter feeder organism, haemocytes and soluble haemolymph factors of this mussel contributed effectively in their defense strategy and survival. Further, deviations from these basal values can be used to predict various stressful conditions. Yet, use of such attributes for monitoring of marine pollutants is rarely attempted. The present study provides seasonal baseline data of 11 relevant haemolymph and mantle fluid parameters to predict the eco-immunological status of apparently healthy, *P. viridis*.

The entire sampling was done three times a year which is based on average temperature and average precipitation, coinciding the spawning season. Relevant immune-biochemical parameters for the haemolymph as well as mantle fluid such as total haemocyte count (THC), superoxide anion generation (SO), phenol oxidase activity (PO), lysozyme activity, glucose content, total protein, albumin, cholesterol, triacylglyceride and were also evaluated for each samples. Condition index evaluated for each season showed an increasing trend in the first two seasons indicating somatic growth, and for other seasons, it was appeared to be comparable. Total bacterial counts peaked in summer season. Lysozyme and albumin were found beyond detection level in the present study. Haemolymph Superoxide anion production showed a decreasing trend among seasons, while it reached maximum at the monsoon season for mantle fluid. Phenoloxidase activity was highest in the monsoon season

for haemolymph and mantle fluid. No significant difference ($P<0.05$) was observed in THC and glucose levels of haemolymph between three seasons. However, mantle fluid glucose showed a peak in summer season. Conversely, no significant difference in haemolymph protein level was observed between the first two seasons, where it differed significantly among seasons for mantle fluid. Circannual trend for haemolymph cholesterol showed a steep decrease in first two season where triacylglyceride indicated an opposite pattern. Conversely, both showed similar pattern in mantle fluid. A reproduction-immunity trade-off is observed for protein, cholesterol and triglyceride in haemolymph with lower value observed for spawning season. However, none of the parameters studied showed a consistent significant correlation pattern ($P<0.001$) with respect to season. Further investigations are required to pave light on the seasonal dynamics of metabolic interaction and their immune outcome through interlinking of these attributes to tissues and body fluids with their active transport in *P. viridis*.

Ferritin, a principal protein found in the cells of almost all living organisms for iron storage and immune responses have rarely been reported in mussels, and to our knowledge *ferritin* gene is not yet fully characterized in *P. viridis*. So this study also aimed to characterize the *ferritin* gene in Asian green lipped mussel. The full length cDNA of *ferritin* gene was found to be 798 bp with a deduced amino acid of 173. It has a Euk_Ferritin domain with three conserved residues- ferroxidase diiron center, ferrihydrite nucleation center and iron ion channel. This result may serve as a reference for further studies regarding the expression of this gene. Thus, the outcome of the study can be exploited in various pollution monitoring studies and disease management strategies for *P. viridis* culture.