Chapter 1

GENERAL INTRODUCTION

1.1. Pearl Oyster Aquaculture

Pearls have been known to the mankind since the beginning of civilization. They are highly esteemed as gems for their beauty and splendor. Pearls are formed when an irritant like a grain of sand is swept into the pearl oyster and is lodged within it where it gets coated by a micro thin layer of nacre, a silvery substance. India has the highest demand for pearls for setting in jewelry, and is particularly famous for its pearl oyster resources which yield superb pearls. The pearl oyster fisheries of India are located mainly in two areas: the Gulf of Mannar off Tuticorin coast and the Gulf of Kutch on the northwest coast. Habitats of these pearl oysters are different viz., at depths up to 23 meters in the Gulf of Mannar and in the intertidal zone in the Gulf of Kutch, and hence have different environments. These bivalves form large beds on hard substrata in the Gulf of Mannar, while they are sparsely distributed in the Gulf of Kutch. The pearl oyster resources of these two areas have been exploited for pearls until the early 1960's.

In October 1972 the Central Marine Fisheries Research Institute started a pearl culture research project at Tuticorin. Success came in July 1973 when a perfectly spherical pearl was produced. This breakthrough was achieved by introducing a graft of the oyster mantle in the gonad of an adult specimen together
with a shell bead nucleus. The CMFRI also succeeded in artificially spawning *Pinctada fucata*, rearing of larvae and producing seed in the laboratory by hatchery techniques. This breakthrough is very important in the light of the difficulty in obtaining sustained supplies of oysters from natural banks for culture purposes. Recently the CMFRI also produced seed of the black-lip pearl oyster, *Pinctada margaritifera* which produces the highly valuable black pearl. The development of the pearl oyster hatchery technology in India in 1981 opened the way for commercial culture of this species. The hatchery is one of the most important source of sustained supply of pearl oysters.

The pearling industry is among the most potentially profitable aquaculture businesses. For example, hundred grams of gem pearls could be valued at more than US$ 2,600 (Anon, 2007). This is valued hundred times than the other aquaculture commodities on a weight basis. Globally, value of cultured pearl produced was estimated at around three billion US dollars (Anonymous, 2014). It has increased by approximately 40% compared to 2003 (Kremkow, 2005). Cultured pearl is contributed primarily by four pearl producing molluscs: (1) freshwater mussels; (2) the black-lip pearl oyster, *Pinctada margaritifera* ; (3) the silver or gold-lip pearl oyster, *P. maxima*; and (4) the Akoya pearl oyster, *Pinctada fucata*.

1.2. Biology of Pearl Oyster

1.2.1 Taxonomy

The true pearl oyster is a bivalve mollusc which belongs to the genus *Pinctada* (Roding) under the family Pteriidae, order Pterioida. Six species of pearl oysters, *Pinctada fucata* (Gould), *P. margaritifera* (Linnaeus), *P. chemnitzii* (Philippi), *P.
sugillata (Reeve), P. anomioides (Reeve) and P. atropurpurea (Dunker) occur along the Indian coasts. The taxonomic position of Pinctada fucata used for the present work is described below.

The taxonomic position of *Pinctada fucata*

- **Phylum** Mollusca
- **Class** Bivalvia
- **Order** Pterioida
- **Family** Pteriidae
- **Genus** Pinctada Roding, 1798
- **Species** Fucata (Hynd 1955)

### 1.2.2 Morphological Features of *Pinctada fucata* (Gould)

The hinge is fairly long and its ratio to the broadest width of the shell is about 0.85 and to the dorsoventral measurement is about 0.76. The left valve is deeper than the right. Hinge teeth are present in both valves, one each at the anterior and posterior ends of the ligament. The anterior ear is larger compared to other species, and the byssal notch at the junction of the body of the shell and the ear is slit-like. The posterior ear is fairly well developed. The outer surface of the shell valves is reddish or yellowish-brown with radiating rays of lighter colour. The thick nacreous layer on the inner surface has a bright golden-yellow metallic luster. Pictorial representation of the anatomy of *P. fucata* is given in Fig. 1.1.
1.3 Life Cycle of Pearl Oyster

Pearl oysters start life as males and change into females after 2-3 years. Each female can release millions of eggs into the water, which are fertilized externally by sperm from the male. (Fig.1.2). Eggs hatch and the oyster pass through various larval phases during which they remain swimming freely in the water. Length of the larval life varies with the temperature, food and other environmental factors. Larva can swim weakly by the use of highly developed velum, although they are generally transported by current and tides. Just before setting, the larva develops a pair of eye spots and a foot. This period is probably most critical in an oyster’s life. Oyster shells are most common culth material, although the larvae will attach to almost any clean, hard surface. At between 25 and 35 days of age, the larvae starts spending more time crawling on the bottom and finally metamorphose into the juvenile pearl oyster that attaches itself to the substrate (Galtsoff, 1964)
1.4 Pearl Formation

Natural pearls are formed as a reaction to an irritant in the internal part of a mollusc (Kunz & Stevenson, 1908; Strack, 2006; Streeter, 1886; Ward, 1995). The irritant may be small particles or parasites trapped or mantle scratches due to friction or predator attack (Strack, 2006; Ward, 1995). However, pearls will not be formed without the existence of epithelial cells from the nacre secreting mantle tissue (Simkiss & Wada, 1980). The epithelial cells begin to proliferate and form a ‘pearl-sac’ to cover the irritant (Taylor & Strack, 2008). The pearl-sac then begins to deposit minerals (nacre) around the irritant as a kind of internal defense mechanism resulting in the pearl (Dakin, 1913; Kunz & Stevenson, 1908). Pearl is produced by the process of bio-mineralization of calcium carbonate crystals. As the deposition continues and the resulting pearl grows. Shape of the irritant is usually irregular and this irregularity causes pearls to grow asymmetrically in shape (baroque type).

1.4.1 Cultured Pearls

Cultured pearls are divided in two types: bead nucleated and tissue nucleated (also called non-nucleated pearls) (Scarratt et al., 2000). Principally, bead nucleated pearls are pearls generated from nuclei and mantle tissue while tissue nucleated
pearls are generated from mantle tissue only. Bead nucleated pearls consist of blisters (Mabe or half pearls), flat or coin pearls (not common) and round pearls (Fiske & Shepherd, 2007; Kennedy, 1998), while tissue nucleated pearls include several types of freshwater cultured pearls and keshi.

1.4.1.1 Mabe Pearl

Mabe pearl is an image pearl produced by placing a hemispherical object or a miniature image against the side of the oyster shell interior. The major advantages of Indian marine Mabes over the ones produced in freshwater mussels are the much shorter gestation period (2 months as compared to 18 months) and the superior quality of the nacre. The process of Mabe pearl formation begins with gluing a special plastic form (nucleus) on the interior of the pearl oyster shell (nucleus size from 8mm to 15mm in diameter). Once glued into the shell, the presence of the nucleus stimulates the pearl oyster to protect itself against the irritation by covering it with nacre, thus forming a Mabe pearl. Once implanted with Mabe pearl nuclei, the pearl oyster should be returned to the farm as soon as possible to minimize stress. There are varieties of ways to hold pearl oysters on a farm. The simplest way is to keep pearl oysters in plastic mesh trays, in bags on a rack or directly on the bottom in a calm, protected area.

1.4.1.2 Cultured Round Pearl

The second type of bead-nucleated pearls is the cultured round pearl which has greater value. Production of round pearls requires a round nucleus to be implanted with a piece of mantle (nacre secreting) tissue from a donor oyster into the
gonad of a recipient oyster. This process is known as ‘pearl implantation’ or ‘grafting’ or ‘seeding’.

1.5 Immune/Defense Mechanisms of Pearl Oyster

The reason for high mortality in pearl oyster is related to ocean pollution, disease outbreaks and stock degeneration (Richard & Jackie, 1995; Potasman et al. 2002). As secondary filter feeders, the bivalves concentrate bacteria, viruses, pesticides, industrial wastes, toxic metals and petroleum derivatives, making them susceptible to disease problems. In order to control the disease and enhance the yields and quality of seawater pearl, it is necessary to carry out research on the innate immune defense mechanisms of the pearl oysters. Although, pathology information has been accumulating, research on bivalve immune systems and the underlying molecular mechanisms are still at an early stage. As an invertebrate, the bivalves rely exclusively on an innate, nonlymphoid system of immune reactions. The internal defense of bivalve is mediated by both cellular and humoral components. The former includes phagocytosis or encapsulation with subsequent pathogen destruction via enzyme activity and oxygen metabolite release, while the latter includes various reactions mediated by a series of molecules. One strategy to combat the problem is to identify defense related/disease resistance genes and use them for genetic improvement of cultured stocks. There are different types of molecules involved in the disease resistance viz. antioxidants, antimicrobial peptides, pattern recognition receptor proteins (PRPs) etc. which are genetically controlled. Among these, the antioxidant enzyme genes and PRP genes have been taken for the present study.
Stress induces the organisms to release reactive oxygen species (ROS), which are natural products of oxidative metabolism and are essential for eliminating invaders (Alves de Almeida et al., 2007). However, excessive ROS can damage a number of cellular macromolecules (Hartog, et al., 2003). Therefore, aerobic organisms require an effective defense system against reactive oxygen species, which are produced following single electron reductions of molecular oxygen. Consequently, organisms contain a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids. (Sies, 1997; Vertuani, et al., 2004). In general, antioxidant systems either prevent these reactive species from being formed, or remove them before they can damage vital components of the cell. (Sies, 1997; Davies, 1995). Function of the antioxidant system is not to remove oxidants entirely, but instead to keep them at an optimum level (Rhee, 2006).

The innate immune system is designed to recognize molecules shared by groups of related microbes that are essential for the survival of those organisms and are not found associated with mammalian cells. These unique microbial molecules are called pathogen-associated molecular patterns (PAMP), and include lipopolysaccharides (LPS) from the gram negative cell wall, peptidoglycan (PG) and lipotechoic acids from the gram positive cell wall. In order to recognize PAMPs, various body cells have a variety of corresponding receptors called pattern recognition receptor proteins (PRPs) which are capable of binding specifically to the conserved portions of these molecules (Raetz et al., 1991; Ulevitch and Tobias, 1995). Once an invading pathogen gain entry into the body of the host, they encounter a complex system of innate defense mechanisms involving cellular and humoral responses.
1.6 Biomineralization

Biomineralization is the formation of minerals by organisms, an extremely wide spread biological process found in organisms from all kingdoms. More than 70 mineral types are known (Mann, 2001). The first recognized scientific studies on biominerals were undertaken by zoologist more than a century ago, describing the various forms of endo and exo-skeletons in vertebrates and invertebrates respectively (Bouligand, 2004). Originally these investigations were descriptive, based primarily on typical crystallography and histology. As a result two major biominerals were catalogued; carbonates in invertebrates and calcium phosphates and carbonates in vertebrates. Furthermore, these biominerals were described with additional orders of complexity, being crystal polymorphism and numerous microstructures in which these polymorphs are arranged (Mann, 2001). Subsequently, the scientific field matured to include collaborations with chemists from which a third order of complexity emerged; the organic matrix component (Crenshaw, 1972; Marin and Luquet, 2004). It has long been theorized that this matrix is the keystone to the unique crystal structures found in biominerals, and through various demineralization processes the organic matrix was extracted and analyzed, the components of which are a mixture of proteins, glycoproteins and polysaccharides (Cariolou and Morse, 1988). Isolation of these components has proven difficult as many matrix characteristics are not amenable to classical fractionation techniques (Miyashita et al. 2000). The recent evolution of molecular-oriented biomineralization research could be used to elucidate the biologically controlled mineralization process.
The pearl oyster shell consists of two mineralized layers— an inner nacreous layer made from aragonite and an outer prismatic layer made from calcite. The mantle epithelium is the organ responsible for shell formation which takes place in the fluid-filled extrapallial space between the mantle and the shell. While the nacreous layer is formed by epithelial cell secretion at the mantle center, the outer prismatic layer is formed by secretion at the mantle edge (Sudo et al. 1997; Wada, 1999; Takeuchi and Endo, 2006). Secretion of organic and inorganic materials from mantle epithelia that condition the composition of the extrapallial fluid is an essential factor for the regulation of shell calcification. More specifically, a macromolecular complex called the organic matrix has been thought to be the controller of shell formation (Lowenstam and Weiner, 1989; Mann, 2001). Organic matrices are of two types, soluble and insoluble, the insoluble matrix provides the framework and mechanical properties for biomineralization, while the soluble matrix is involved in mineral nucleation and growth (Mann, 2001), thereby, possibly controlling calcium carbonate polymorphism (Belcher et al. 1996; Falini et al. 1996; Feng et al. 2000).

1.6.1 Genetic basis of Pearl Formation

Biomineralization processes leading to the formation of pearl in molluscs is controlled by specialized proteins. These proteins are regulated by specific genes encoding them. There is a paucity of sufficient information on the functional genes involved in pearl formation; therefore, an attempt was made to characterize some of them. In the present study, Mabe pearl was taken for gene expression study instead of round pearl because of the short gestation time for pearl formation.
Therefore, the present study was directed primarily towards the identification and characterization of functional genes involved in the defense and pearl formation in pearl oyster, *P. fucata*. Secondly, to evaluate the expression of these genes.

**Objectives of the study**

a) Identification and characterization of the following functional genes of pearl oyster.

   i. Antioxidant genes: superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione-S-transferase (GST).

   ii. Pattern recognition receptor proteins (PRP) genes: F-type lectin, galectin and lipopolysaccharide and β-1, 3-glucan binding protein (LGBP).

   iii. Pearl forming genes: nacrein, prismalin-14 and N19.

b) Expression analysis of the above defense related and pearl forming genes.

   i. Gene expression of antioxidant genes and PRP genes following exposure to lipopolysaccharide

   ii. Expression of pearl forming genes following Mabe implantation.