Luminescent Bacteria in Aquaculture

Aquaculture is the fastest growing food sector globally has been established itself as high protein resource to fulfill the food demand as the natural resources exhibits under over exploitation. But, presently, the biggest problem faced by the aquaculture industry worldwide is on diseases due to various biological and non-biological agents. Among the groups of microorganisms causing serious losses in shrimp culture, the best known are bacteria because of the devastating economic effects they have on affected farms. Bacterial diseases, mainly due to *Vibrio,* have been reported in penaeid shrimp culture systems implicating at least 14 species and they are *Vibrio harveyi, V. splendidus, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. campbelli, V. fischeri, V. damsella, V. pelagicus, V. orientalis, V. ordalii, V. mediterrani, V. logei* etc. Luminescent vibriosis due to *Vibrio harveyi* had been an important disease causing mass mortalities in hatchery-reared larval *Penaeus monodon*, but its occurrence has not been rampant in cultured juveniles in grow-out ponds until the last quarter of 1993. In 1994 luminescent vibriosis caused mortalities and termination of grow-out activities especially in the first 45 days of culture (DOC). To some extent, these disease problems occurred because the interactions of microbes and their effects on animals and their environment. The common name,

luminescent vibriosis, given to the disease is actually a description of the main symptom observed in affected individuals when observed in the dark. Shrimp in affected ponds were found near the edge of the ponds and swam with their heads poised near the water surface. They, thus, appeared as if they were swimming vertically and floating. Gross examination of these shrimps reveals no significant lesions on the shell or its underlying tissues. Occasionally, the cephalothorax region in the area of the hepatopancreas appeared dark in colour.

Luminous vibriosis is presently a major disease problem in grow-out shrimp culture. The disease usually occurs during the first month of culture and can cause more than 50% mortality. Luminous *Vibrio,* the causative organism of the disease, is an opportunistic pathogen. They can invade the host through the hepatopancreas, a common target organ of most bacterial pathogens of shrimps. These bacteria proliferate and colonize in the host's digestive tract and become pathogenic. Thus, it is necessary to determine the bacterial load of the hepatopancreas to assess the levels of bacteria, specifically luminous *Vibrio,* that can be tolerated by the shrimp host.

Vibrio harveyi a pathogen of *P. monodon* that causes severe losses, is luminescent, a non-sucrose fermenter and its colonies are green on the TCBS agar, is a normal flora of warm near-shore marine waters. They are commonly termed as the 'free-living' bioluminescent bacteria which can attach to surfaces of marine crustaceans or can be ingested by the host and establish residence in the gut.

Luminescent bacteria were found in the midgut contents of *P. monodon* spawners and pond-reared juveniles. The high abundance of luminescent *Vibrio* is consistent with occurrence of disease and poor or zero harvest results.

The most commonly reported association between aquatic invertebrates and gut microbes is that of ingestion of bacteria. Thus, luminous *Vibrio* present in the environment can enter the shrimp host through ingestion of feeds and rearing water loaded with high numbers of the pathogen. The occurrence of mortalities in *P. monodon* juveniles affected with luminous vibriosis is preceded by the dominance of *V. harveyi* in the pond-rearing water. Thus, continuous ingestion of luminous bacteria by the shrimp host may initiate the colonization of this pathogen in the digestive tract.

Luminous bacteria dominate the *Vibrio* population of the shrimps' hepatopancreas affected with disease. Moreover, the levels of luminous bacteria were higher in diseased than in healthy shrimps. This indicates the proliferation of the pathogen in the hepatopancreas, which eventually seize control of the digestive tract and become pathogenic to the shrimp host. Moreover, colonization of the hepatopancreas by luminous bacteria can replace the other normal flora of the gut, which have beneficial association to the shrimp host, such as increasing digestive efficiency. Luminous *Vibrio* can cause severe damage to the hepatopancreas of the shrimp host, such as inflammatory response in the intratubular sinuses. This may then lead to physiological imbalances and stress in the host causing slower growth and eventually mortalities. Mortalities resulted from a massive invasion of the hepatopancreas resulting to a reaction whereby the whole hepatopancreas became inflamed. This pattern was seen in shrimp juveniles, which succumbed to infection at below 45 DOC. The disease takes a chronic course in juveniles (> 45 DOC) and slow growth occurs if only a few tubules are affected thereby causing dysfunction. This dysfunction results from the nodule or granuloma-like formation around affected tubules resulting from fibrosis and melanin deposition. Recurrent outbreaks of mortality did occur when infection takes this course because shrimps that were unable to cope with continuous and lingering sickness eventually succumbed to infection and died. The histopathology of these animals also showed lesions in the lymphoid organ.

The available information explains regarding the occurrence and severity of luminous bacteria in shrimp. The shrimp broodstock shed its midgut contents into the water almost simultaneously with the eggs during spawning are suspected to be the main source of luminescent vibrio. The coastal water may also be a major source of infection because luminous bacteria are found abundantly in marine and estuarine habitats.

It shows that *V.harveyi* has emerged into a devastating pathogen of penaeid larvae, especially those of the black tiger shrimp. However, previous studies have also indicated that it is a normal constituent of the non-pathogenic flora of marine animals. All these luminous bacteria, whether normal flora or pathogens, can exhibit bioluminescence.

Luminous disease was reported initially from *Penaeus monodon* larvae at naupliar to PL stages in hatcheries. Since then, a rising incidence of luminous disease that can cause mortality to the larval stock has occurred in hatcheries and grow-out farms. *V. harveyi* was predominant the isolates and followed by *V. spledidus*, *V. fischeri* and other luminous species.

However, vibriosis causes mortality in larvae, postlarvae, juveniles, subadults and also adults. At times, outbreaks result in mortality up to nearly 100% of affected populations. The gross signs of localized infection in the cuticle or subcuticle are called shell disease or black or brown spot disease and these superficial infections develop into systemic infections under some circumstances. Finally, it is the systemic infection that causes mortality. Any wound or rupture on the exoskeleton is a good site for luminous bacterial infection.

The disease outbreaks are reported to be initiated by sudden changes in coastal water due to monsoon rains or cyclones. The problem affected most farms located along coastal and brackishwater rivers with multiple users including agricultural, industrial, domestic and aquacultural ventures. Species such as *V. harveyi* are universally present in coastal water and appearance of pathogenic or luminous forms is often associated with changes in salinity, temperature or nutrients, some of which can be induced experimentally. Results from experimental bath infections confirmed that zoea and mysis stages are more susceptible than postlarvae. The studies also suggest that they induce the disease more frequently in dry than rainy season and it is mostly related to salinity levels or effluents from culture operations. It was found that alkalinity affects luminescence expression. Higher than the optimal pH media gives strong luminescence. Mostly *V. harveyi* has been confirmed to be a pathogenic agent associated with shrimp mortality. Experimental studies proved that an intramuscular injection of luminescent *V. harveyi* strain in combination with a bacteriophage is fatal to juvenile shrimp.

Earlier studies indicated that the bacterial pathogenesis results in mortalities up to 100% for nauplioid to zoeal stages. Living or dead shrimp larvae and even the seawater in disease outbreak areas exhibits luminescence in the dim light. Other gross features of the diseased shrimp are milky white bodies, weakness, swimming disorders and loss of appetite eventually leading to death.

Using luminous media (LM), luminescent bacterial colonies can be isolated from the diseased specimens as well as from the hatchery rearing water. These pathogenic forms multiply in intestine and enter the hepatopancreas for further multiplication and then invade the blood and liberate toxins. Hence, the infected shrimp shows a bluish hue and emits bioluminescence. The movement of shrimps in groups looks like lights moving in the dark. This is due to the electron transport that proceeds by reaction of luciferase enzyme which catalyzes the interaction amongst reduced flavin mononucleotide ($FMNH_2$), oxygen and a long chain mononucleotide (FMN) and also aliphatic carboxylic acid emitting the light. Virulence is mostly associated with extracellular products (ECPs) including proteases, haemolysins and cytotoxins, which allow the bacteria survive and replicate within the host tissues. The intensity of bioluminescence is directly proportional to the luminescent Vibrio bacterial density. There are some chronic carriers, which cannot be eliminated from the larval cultures. When they die or eaten by others, a sudden multiplication of the bacteria occurs causing luminescence and this happens particularly when the shrimp larva experiences any stress due to physicochemical or environmental conditions.

Vibriosis in penaeids is generally recognized as a secondary infection influenced by factors such as stress, environmental failures and high numbers of potentially pathogenic bacteria. Continuous exposure of shrimp postlarvae to high numbers of *V. harveyi* in the environment can easily cause primary vibriosis as the bacteria are able to multiply on shrimp surfaces. The differences in the response of various shrimp populations to factors triggering vibriosis was due to their individual capacity to resist stress influenced by their nutritional and physiological make-up. It is thus good practice to select postlarvae of good quality and which meet criteria set by industry experience.

Control of luminous Vibrio

One of the immediate mitigating measures used by the shrimp industry to solve problems with high bacterial loads in the source was the use of reservoirs, where effective reduction of bacterial population can be done using sedimentation and treatment.

Presently, the industry uses water disinfectants such as various forms of), benzalkonium chloride (Bionex-80) and other chemicals to conditions the water before use. Reduction in potentially harmful bacterial populations was achieved in the reservoir. Effective water treatment and conditioning is necessary and these can be further enhanced with the use of reservoirs.

Stocking of shrimp postlarvae in pond water with 10^2 luminescent bacterial cells/ml should be avoided as pathogenicity tests using postlarval *P. monodon* showed that significant mortalities occurred upon exposure to 10^2 *V. harveyi* cells/ml within 48 h. From the results obtained in bacterial quantification, a threshold level of 10^4 CFU /hp and a safe level of 10^2 CFU /hp luminous bacteria within the first 30 DOC can be deduced. Thus, luminous bacteria should be maintained below this level or within the safe level to prevent infection. However, this will depend largely on the luminous bacterial load of the rearing water, which is the main source of the pathogens entering the host. Hence, luminous bacterial level in the pond rearing water should be maintained below 10^2 CFU / ml to avoid infection of the shrimp host.

Bacterial septicemia that has resulted either directly or indirectly from relatively long-term stress is one of the reasons for shrimp mortality. The environment decides the crucial balance between the host and pathogen. In many a situation, the shrimp under culture live healthy normal life even in the presence of pathogens but when environmental stressors occur and the balance tips in favour of the disease. Any imbalance in the microbial flora of rearing water or in the gastrointestinal tracts of shrimp may lead to pathogenesis. Shrimp larvae undergo stress due to factors such as inadequate physicochemical and microbiological quality of water, inferior nutritional status and high stocking densities. To control luminous vibriosis and other diseases, it is important to follow the appropriate management techniques that improve sanitation or reduce culture stressors.

Proper sanitation, cleaning and drying of culture tanks are essential every time before use to reduce pathogenic infections. Rigorous water management methods must be adopted to prevent the entry of luminescent vibrios through culture water. To achieve this, chlorination, UV sterilization and ozonation are used in hatchery systems. The use of filtered seawater through 3µm pore size allows only the normal bacterial communities to remain and it has resulted in better survival and growth rates of cultured shrimp larvae.

The indiscriminate use of antibiotics as a control measure against luminous vibriosis, which has resulted in drug resistant bacteria. The chlorination would not result in elimination of vibriosis present in the sediments completely and therefore repopulation of the pathogen occurs immediately after dechlorination in the culture systems.

Pre-sterilization of larval culture water is not always effective as opportunistic pathogens rapidly recolonize cultures. *V. harveyi* can form biofilm on various surfaces such as plastic, cement, stainless steel etc. and such biofilm cells are resistant to treatment of antibiotics and chlorine even at 100 ppm level. The biofilms formed by the bacteria are reported to be protected by antibacterial agents making the treatments ineffective and these biofilms cause anoxic conditions, producing hydrogen sulphide and ammonia, which again are detrimental to growing shrimp larvae. Such biofilm bacteria resistant to antibiotics and antimicrobial agents may be a cause for the present virulence problems in shrimp hatcheries.

Thus it is evident that it is not possible to eliminate luminescent vibrios by use of sanitizers and chemicals. After harvesting, treatment of the larval rearing facilities with a flush or vigorous spray of freshwater has been recommended as a routine practice. “All in-all out” batch culture punctuated by thorough disinfection and drying has to be adopted as a general practice to manage the pathogens.

Avoidance of stress is one of the most important ways to prevent disease. The culture species should be well fed, not over-crowded and not handled excessively. Good water quality must be maintained. An easy way to avoid stress is to stock the culture species in low numbers and to use small amounts of feed. Although luminous pathogens exist naturally in the water, they certainly need carbon and nitrogen (C+N) for growth and multiplication. Thus, the first action to be taken to reduce the pathogen is not to overfeed the shrimp as the leftover nutrients in the tanks will be helpful for the multiplication of pathogen bacteria. To prevent environmental stress to the animals, it would be important to degrade the toxic substances (waste products). Microorganisms are highly versatile and can utilize toxic substances such as ammonia and hydrogen sulphide and convert them to less harmful nitrate and sulphate.

The technology of competitive advantage using benign bacteria, popularly known as bioaugmentation or probiotic approach, is also finding its way in shrimp aquaculture through the application of bacteria-enzyme preparations to prolong the dominance of harmless bacteria and give the newly-stocked postlarvae a 'healthy environment' while they are still young and relatively more susceptible. The concept of cleaning the environment using microorganisms is giving fruitful results. The term 'bioremediation' is often used to describe the process of converting the environmental parameters to favourable levels using microorganisms. These microorganisms, which are actually probiotics can eliminate pathogens by competitive exclusion and could become important tools in disease management in shrimp culture.

Probiotic bacteria secrete many enzymes that degrade slime and biofilms of vibrios. The enzymes break down not only the slime layers around gram-negative bacteria but also utilize the organic matter and make the nutrients unavailable to the growing vibrios.

The use of beneficial bacteria (probiotics) to displace pathogens by competitive processes is being used in the animal industry as a better remedy and is now gaining acceptance for the control of pathogens in aquaculture.

The term 'probiotic' from the use of live microorganisms in feed, to "a probiotic is a mono-or mixed culture of live microorganisms that, applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora". In aquaculture, it is clear that it also applies to the addition of live bacteria to tanks and ponds in which the animals live, because these bacteria modify the bacterial composition of the water and sediment. The health of animals is thus improved by the elimination of pathogens or at least minimizing the effect of pathogens and by improving water quality. Therefore, probiotics are used in aquaculture not only as feed supplements, but also as water additives.

More recently researchers have sought beneficial microbes for shrimp aquaculture by attempting to isolate them from seawater, sediments and gastrointestinal (GI) tracts of marine animals.

These are capable of producing antimicrobial substances that can inhibit the growth of pathogens. It has been reported that both beneficial bacteria and unicellular algae capable of inhibiting pathogenic bacteria. The isolated Bacillus strain from the gastrointestinal tract of *Penaeus monodon* broodstock inhibited the luminescent disease bacterium *V. harveyi* and could promote better survivals of black tiger shrimp larvae.

The farms, which used the probiotic bacteria, had either a very low abundance or a complete absence of luminous *Vibrio* in pond water and very good harvest results. Furthermore, luminescent *Vibrio* were completely absent at all stages of grow-out from the pond sediment in the presence of the probiotics. It was found to be important to have regular additions of the probiotics. The research findings indicate that the *Vibrio* populations were being affected by the probiotics that were added. This change was beneficial to the shrimps, as they were protected from vibriosis. Shrimps were healthier in ponds with probiotics.

Unlike land animals, water media that supports their pathogens independently of the host animal surrounds aquatic farmed animals and so the pathogens can reach high abundances around the animal.

Vibrio grow attached to algae, and may reach high population densities after being ingested with the algae and then excreted with lysed algae in fecal pellets by zooplankton; they are gut bacteria in fish, prawn and shrimp as well as zooplankton. In aquaculture ponds, where animal and algal population densities are high, *Vibrio* numbers are also high compared to the open sea. The onset of prawn disease due to exposure to these high numbers of *Vibrio*, especially when pathogenicity has increased indicates that a defense is needed. Hence, probiotics are used to control *Vibrio* or preventing attachment.

Probiotics such as the use of *Bacillus* spp. described here for sustainable aquaculture. There are several reasons why it is better to add *Bacillus* spp. rather than antibiotics to control *Vibrio* species. Many different antibiotic compounds are naturally produced by a range of *Bacillus* species. Other bacteria are unlikely to have resistance genes to all antibiotics at one time, especially if they have not been exposed to the *Bacillus* spp. previously. *Bacillus* spp. secrete many enzymes that degrade slime and biofilms and allow *Bacillus spp.* and their antibiotics to penetrate slime layers around Gram-negative bacteria.

Furthermore, *Bacillus* spp. compete for nutrients and thus inhibit other bacteria from growing rapidly. Thus any resistant bacteria cannot multiply readily and transfer resistance genes. *Bacillus spp.* also compete for space on surfaces e.g., the gut wall and displace other bacteria if they are present in high numbers.

Because there are many different mechanisms involved in the probiotic process of competition and exclusion, it is difficult for the pathogens to evolve all the necessary resistance genes together. Competitive exclusion is one of the ecological processes that can be manipulated to modify the species composition of a soil or water body or other microbial environment. Small changes in factors that affect growth or mortality rates will lead to changes in species dominance. Microbial ecology and biotechnologies have advanced in the last decade, to the point that and technologies are available for treating large areas of water and land to enhance population densities of particular microbial species or biochemical activities.

The luminescent bacterium utilizes an N-acyl-homoserine lactone (AHL) signaling system to activate the transportation to swarming phase, which produces luminescence, exoprotease and/or virulence. Since a small number of genes are involved, it may be possible to block the AHL pathway by using AHL mimics, which will bind to the bacterial AHL receptors and prevent inter-bacterial signaling that results to inhibit the expression of virulence.

It has been shown that the marine alga *Delisea pulchra* inhibits fouling by the production of furannoses which interfere with AHL mediated expression of bioluminescence, swarming, motility and exoenzyme synthesis in different bacterial species. However, both the production of AHL analogues or mimics are widespread amongst the marine algae and they prevent swarming and virulence in *V. harveyi*. It is possible that these compounds may be used to control bacterial populations by incorporating in microencapsulated diets (MED) for penaeid larval cultures.

The most important strategy to protect shrimp larvae against luminescent vibriosis appears to be by enhancing their natural defense system. Harnessing the host's specific and non-specific defense mechanisms for controlling diseases has considerable potential for health management in shrimp larviculture. This will help to reduce stress from handling and environmental manipulation in order to control disease expression under culture conditions. Frequent health control measures will permit the detection of shrimp immunodeficiencies. This will in turn assist to develop the strategies to decrease the disease susceptibility. Important biotechnological interventions are being developed in the field of immunostimulants and modulators in an effort to reduce shrimp's susceptibility to disease.

Immunostimulants and non-specific immunoenhancers are being incorporated into diet to provide added protection to the animals. Glucans, the cell wall components of many fungi and yeast, are routinely incorporated in vaccines, feeds, and have been reported to show an impact on shrimp immunization. β-glucan treatment enhances disease resistance in tiger shrimp larvae.

It is also found an effective protection against vibriosis through the use of β-1,3-glucan as an immunostimulator. Glucan treatments seem to show an immunostimulatory effect up to 18 days post-treatment. However, the growth cycle of penaeid shrimps is usually over 4 months, which means that glucan treatment will only be effective if repeatedly applied. The glucan-treated post-larvae, juveniles and adults will have a higher average survival.

These findings draw attention to the following risk factors in the development of luminescent vibriosis in newly stocked shrimp juveniles in ponds:

- Abundant luminescent *vibrio* in the source for rearing water and the dominance of this bacterial type within the system;
- Age of postlarvae at transfer from hatchery to stocking in ponds. Although most farms stock PL 18-20, some farms stock them at a younger age;
- Duration of continuous exposure to high population of luminescent vibrios;
- Unavailability of acceptable food for the postlarvae upon stocking. The histological samples show that the most severely affected shrimps are the smallest in the group. Starvation is an obvious precursor of the infection.

There are other risk factors to fully understand the course of luminescent vibriosis in grow-out farms an epidemiological approach to the problem has to be adapted. The presence of viruses, which are known to be commonly associated with postlarvae should be investigated as damage they cause could create portals of entry for opportunistic pathogens. Such study should also come into the practices in the hatchery phase of shrimp culture to understand the resistance or susceptibility of postlarvae to diseases and re-assess the importance of a weaning period or nursery phase to condition hatchery-reared PLs to a relatively harsh grow-out environment.

In addition to these luminous *Vibrio*, there are also other luminous creatures in the coastal waters. These are dianoflagellates particularly *Noctiluca* sp. These dianoflagellates excretes chemical substances, which will have toxic effect on the culture organisms. In aquaculture ponds with high densities of dianoflagellates will reduce the water colour by minimizing the phytoplankton (algal blooms) density due to heavy consumption of nutrients.