CHAPTER - III

Determination of LD$_{50}$ of *Aeromonas hydrophila* and Antimicrobial activity of probiotics against *A. hydrophila*

Introduction

Drug resistance and poor growth in farmed fish are major constraints in aquaculture industry. The immunostimulants hold the promise to improve fish growth and subsequently control fish diseases in aquaculture. The aquaculture industry is having many disease problems. In intensive aquaculture, heavy mortality is considered as a major constraint due to infectious bacterial diseases (Balboa *et al*., 2007). Hence prevention and control of the diseases have been concentrated in good husbandry practices and the use of vaccines and antibiotics have been recommended. Even though vaccines and antibiotics are being developed and used, no antibiotic is used on regular basis as either standard prophylactic or therapeutic measure in aquaculture, since treatment or feeding with antibiotics paves way for the development of resistant bacteria (Ringo *et al*., 2004). Hence an alternative way to antibiotics is the use of probiotics that inhibits the entry and colonization of pathogenic bacteria by producing antimicrobial compounds and thereby supporting the natural host microbial defence mechanism (Gram and Ringo, 2005; Verschuere *et al*., 2000; Gomez *et al*., 2000; Ringo, 1999; Hansen and Olafsen, 1999; Gatesoupe 1999; Sugita *et al*., 1998).

Smith *et al.* (1994) reviewed the results of several studies performed all over the world on sensitivity and resistance of bacterial pathogens of aquatic species to a variety of antibacterial agents. In fish, as in other aquatic organisms, the whole microorganisms have been mainly administered by bacterial species in the form of feed additives. It has shown an improvement in the intestinal microbial population
and the healthy status of fish by colonizing the gut and acting as antagonistic agents to pathogens and so increasing resistance to pathogens (Gateoupe, 1999; Fuller, 1989; Tannock, 1997). Plump (1997) reported that vaccines are not able to completely eliminate pathogens or prevent the target organisms from being present in vaccinated populations. Thus in order to treat the pathogen, several antimicrobial agents such as Amoxicillin, Ampicillin, Chloramphenical, Erythromycin, Flumequineoxolinic acid, Oxytetracycline, Nitrofurozone, Sulphadiazine trimethoprim and Tetracycline (Toranzo et al., 1991; Bakopoulos et al., 1995; Sano, 1998) have been used. However, over the last decade, drug resistant strains carrying a transferable R-plasmid have developed (Takashima et al., 1985; Kim et al., 1993) making treatment with antimicrobial chemotherapeutics was less successful. For a treatment to be effective, microbial susceptibility experiments should be carried out to evaluate the susceptibility and resistance development of antimicrobial agents.

The common microflora present in fish viz., lactic acid bacteria (Gatesoupe, 1994; Jborn et al., 1997), Fluorescent pseudomonads (Bly et al., 1997; Gram, 1993; Smith and Davey, 1993), Coryne bacterium divergens (Mazurkiewicz et al., 2007) Bacillus sp., (Vaseeharan and Ramasamy, 2003) and Yeast Saccharomyces cerevisiae (Irianto and Austin, 2002) are capable of inhibiting the fish pathogens by in vitro assay. On the other hand, it has been reported that the aquatic bacteria isolated from various sources play an important role by their antagonistic effects (Sugahara et al., 1988; Nair and Simidu, 1987; Dopazo et al., 1988). For instance, Bacillus sp., exhibit antagonistic effects against pathogenic microbes causing disease in fish and shell fish (Gatesoupe, 1999; Rengipipat et al., 2000). Sharmila et al. (1996) have reported that since Bacillus sp., constitute a larger part of microorganisms of the gills, skin and intestinal tract, they can persist for a relatively long term and inhibit the entry of
pathogens. *Bacillus sp.*, have been used as biocontrol agents to reduce *Vibrio sp.*, in shrimp culture (Skjermo and Vadstein, 1999, Rengipipat *et al.*, 2000). Many bioactive and pharmacologically active substances extracted from marine algae were reported to exhibit antibacterial activity against fish pathogens (Siddhanta *et al.*, 1977; Mahasneh *et al.*, 1995).

Several testing methods, including Disc diffusion, Broth microdilution, Agar dilution and the E-test (AB Biodisk, Solna, Sweden) have been used to determine the *invitro* susceptibilities of pathogenic bacteria to antimicrobial agents (Huang *et al.*, 1992). Byers *et al.* (1986) have shown that *A. hydrophila* can produce siderophores that confer resistance against the ability of serum transferring to inhibit bacterial growth. Many studies have been attempted further to describe the virulence mechanisms of motile aeromonads. Kou (1973) found that many of the virulent, avirulent, and attenuated aeromonads possessed hemorrhagic factors and lethal toxins. Oliver *et al.* (1981) indicated that both *A. hydrophila* and *A. sorbia* produced enterotoxins, dermonecrotic factors and haemolysins. The present investigations was attempted to determine the virulent dose (LD50) of *A. hydrophila* on healthy *C. striatus* and to examine the antibacterial susceptibility using probiotics against *A. hydrophila*.

**Materials and methods**

*Aeromonas hydrophila*

*Aeromonas hydrophila* is a ubiquitous, free living, heterotrophic, Gram negative bacterium mainly found in areas with a warm climate (Plate 3). It is a motile bacillus with single flagellum that ferments glucose with or without the production of gas. It is prevalent in aquatic habitat with cosmopolitan distribution. It is an opportunistic pathogen that has resulted in heavy mortality in farmed and wild fishes. This bacterium can also be found in fresh, salt, marine, estuarine, chlorinated and
unchlorinated waters. This bacterium can digest materials such as gelatin and hemoglobin.

**Growth study of Aeromonas hydrophila**

The growth pattern of selected A. *hydrophila* was studied in detail. *A. hydrophila* is easily cultured using *Aeromonas* isolation agar (Himedia). Growth of the bacterial cells was measured by direct count using Haemocytometer and Total plate count method (Lakshmanan *et al*., 1971). The number of cells was calculated after measuring the sample intensity or cell count at intervals of 0, 3, 6, 9, 12, 15, 18, 24, 28, 48 and 72 hrs after inoculation of the cells in the fresh medium and the cells were harvested by centrifugation at 5000rpm for 15 min. The pellet was serially diluted and total count was made using Neubaur counting chamber. For viable count 0.1 ml from the dilution, was spread plated on agar plates, incubated at 37°C for 24 hrs and the colonies were counted.

**Determination of LD$_{50}$ of A. hydrophila on Channa striatus**

Experimental fish were randomly selected and distributed into 3m x 1.5m x 1m cement tanks filled with well water at the stocking rate of 10 fish per tank separately for *A. hydrophila* treatments. Triplicates were maintained for each treatment for a period of 10 days and mortality was recorded.

To find out the LD$_{50}$ of *A. hydrophila* on test fish, 18 hrs old broth culture (logarithmic phase) containing different loads of bacteria in physiological saline (0.85% NaCl; pH 7.2) were injected intraperitoneally (Dhanaraj *et al*., 2008). Ten fish from each experimental group were administered with $10^3$ to $10^9$ cells of *A. hydrophila*. The LD$_{50}$ was calculated following Reed and Muench (1938). The fishes were observed carefully for visible external symptoms and behavioral changes. Time taken to lose balance and mortality of the challenged fish was recorded and
death due to *A. hydrophila* was confirmed by reisolation of organism from the liver, spleen, body fluids and intestine.

**Antagonistic activity of probiotic bacteria against *A. hydrophila* (Disc diffusion method)**

A cell free supernatant of each probiotic strain $10^6$ cfu/ml, (*Bacillus subtilis*, *Bacillus coagulans*, *Bacillus licheniformis*, *Saccharomyces cerevisiae*, *Lactobacillus acidophilus* and *Lact-act*® (commercial probiotic, Poseidon Biotech, Chennai.) was obtained by centrifuging at 5000rpm for 10 minutes followed by filtration through 0.20 μm Millipore membrane. Blank sterile discs of 6mm diameter were dipped into the cell free supernatant and dried in an incubator for 15 minutes at 37°C. The impregnated discs were placed on sterilized Muller Hinton agar plate which has a lawn inoculated with 0.1ml of *A. hydrophila*, ($10^6$ cfu/ml). Two probiotic and one control were tested per plate in triplication. The diameter of the inhibition zones around the disc was measured and recorded.

**Data analysis**

The results are presented as mean ± standard deviation. The SPSS statistical program (version) was used for data analysis.

**Result**

*A. hydrophila* injected *Channa striatus* showed reddening at the site of infection and changes were noticed after 9hrs and 10hrs of injection of $10^8$ cfu/ml and $10^9$ cfu/ml concentrations. *A. hydrophila* concentrations of $10^9$ cfu/ml and $10^6$cfu/ml injected fish showed 100 % and 96.96 % mortality. They produced extensive blanching lesion with furuncle like ulcerated core (Plate 3) and at the end of trial they lost the layers of skin and all the individuals died. *C. striatus* injected with $10^7$cfu/ml *A. hydrophila* showed 88.46% mortality and severe lesions were noticed in the
infected portions. The injured tail showed reddish patches and loss of skin layer was observed. $10^6$ cfu/ml, $10^5$ cfu/ml and $10^4$ cfu/ml of *A. hydrophila* injected fish showed cumulative mortality of 71.4%, 44.4% and 10% respectively. They showed slight lesions and swelling on the infected portion. No mortality was found in $10^3$ cfu/ml injected fishes. The determined LD$_{50}$ was $6.2 \times 10^6$ cfu/ml for *C. striatus* (Table 3.1).

**Table 3.1. Determination of LD$_{50}$ for virulent *Aeromonas hydrophila* on *Channa striatus* by intraperitional route (Reed and Muench, 1983)**

<table>
<thead>
<tr>
<th>No. of bacterial cells (cfu/ml)</th>
<th>Number of fish</th>
<th>Died</th>
<th>Survived</th>
<th>Dead ratio</th>
<th>Survival ratio</th>
<th>Mortality Fish Mortality/ hr</th>
<th>Mean Death Time/hr</th>
<th>Cumulative mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^9$</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>42</td>
<td>0</td>
<td>42/42</td>
<td>10/24</td>
<td>100</td>
</tr>
<tr>
<td>$10^8$</td>
<td>10</td>
<td>9</td>
<td>1</td>
<td>32</td>
<td>1</td>
<td>32/33</td>
<td>9/24</td>
<td>96.96</td>
</tr>
<tr>
<td>$10^7$</td>
<td>10</td>
<td>8</td>
<td>2</td>
<td>23</td>
<td>3</td>
<td>23/26</td>
<td>8/48</td>
<td>88.46</td>
</tr>
<tr>
<td>$10^6$</td>
<td>10</td>
<td>7</td>
<td>3</td>
<td>15</td>
<td>6</td>
<td>15/21</td>
<td>7/72</td>
<td>71.4</td>
</tr>
<tr>
<td>$10^5$</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>8</td>
<td>10</td>
<td>8/18</td>
<td>6/72</td>
<td>44.4</td>
</tr>
<tr>
<td>$10^4$</td>
<td>10</td>
<td>2</td>
<td>8</td>
<td>2</td>
<td>18</td>
<td>2/20</td>
<td>2/96</td>
<td>10</td>
</tr>
<tr>
<td>$10^3$</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>28</td>
<td>0/28</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Proportionate distance** = \( \frac{\text{Mortality above 50\%-Mortality below 50\%}}{\text{Mortality above 50\%-Mortality below 50\%}} \)

\[ = \frac{71.4-50}{71.4-44.4} \]

\[ = \frac{21.4}{27} = 0.79 \]

\[ = \text{Dilution above 50\% + Proportionate distance} \]

\[ = 6+0.79 = 6.79 \]

\[ \text{Anti log 6.79} = 6.2 \times 10^6 \]

\[ \text{LD}_{50} = 6.2 \times 10^6 \text{ cfu/ml} \]
Discussion

In the present study severe lesions were observed following *A. hydrophila* administration (10^9 cfu/ml) during the LD_{50} assay. Similarly Lio *et al.* (1998) stated that injection of *A. hydrophila* intraperitonially at a concentration of 10^9 cfu/ml induced severe dermomuscular necrotic lesions in both cat fish and snakeheads. In the present study *A. hydrophila* was used for *in vitro* study and determined that LD_{50} was 6.2x10^6 cfu/ml. Similarly Khail and Mansour (1997) found that *A. hydrophila* was found to produce haemolytic and proteolytic exotoxin that are lethal to tilapia and the LD_{50} value was 2.1 x 10^4 cells/fish. The lethal effect was also attributed to the unknown virulent factors that were responsible for 20% mortality. Lipton (1987) observed that *P. aeruginosa* had a lethal dose of 1.5x10^5 cfu/ml for *C. carpio* and 4.2 x10^5 cfu/ml for *O. mossambicus*. Dhanaraj *et al.* (2008) have reported LD_{50} of *Vibrio sp.* as 10^5 cfu/ml, *A. hydrophila* *A. salmonicida* and *E. coli.*, as 10^6 cfu /ml in Channa striatus. Manohar (2005) reported the LD_{50} of *A. hydrophila* as 10^6 cfu/ml in *C. carpio*. These reports support our present findings.

The antibacterial effects of bacteria were generally due to either individual or combined production of antibiotics, bacteriocins, siderophores (Gram and Melchiorse, 1996), lysozymes, proteases and alterations of pH by organic acid production etc (Sugita *et al.*, 1998). The inhibitory effects of such compounds are highly dependent on the experimental conditions and vary with *in vitro* and *in vivo* conditions (Gatesoupe, 1999). This is mainly due to the invasive patterns of pathogens. Several studies have proved that fish skin and gills are the major site for proliferations for pathogen invasion (Kanno *et al.*, 1989; Spanggaard *et al.*, 2000). Olsson *et al.* (1992) have stated that the intestinal tract is a portal of entry for pathogens in host. Several authors have reported the pathogenic activity of *Vibrio sp.*
Aeromonas sp., Edwarsiella sp. and Yersinia sp. in vitro studies (Dopazo et al., 1988; Ruiz et al., 1996; Sugita et al., 1996; Byun et al., 1997; Gibson et al., 1998 and Das et al., 2005).

Disc diffusion method was employed in the present study and the degree of inhibition of different probiotics against pathogen was assessed. Lact-act® produced better zone of inhibition followed by B. subtilis and S. cerevisiae. In the present investigation B. subtilis, B. coagulans, L. acidophilus S. cerevisiae B. licheniformis and Lact-act® produced zone of inhibition against A. hydrophila. Among the probiotics Lact-act® (14 ± 0.2mm) and B. subtilis (12 ± 1.0mm) produced maximum zone of inhibition against A. hydrophila. Sugita et al. (1998) reported that Bacillus NM 12 had antibacterial spectrum against intestinal bacteria of coastal fish due to the production of heat labile siderophore substances. Our present finding is supported by these reports. Bacillus sp. has produced secondary metabolite to extracellular compounds such as bacteriocin, hydrogen peroxidase and other organic acids and also produced inhibited pathogenic bacteria in fish and shell fish by successful colonization in the gut of the host (Daeschel, 1989; Gatesoupe, 1999; Irianto and Austin 2002). Skjermo and Vadstein (1999) and Rengipipat et al. (2000) also reported that the Bacillus spores have been used as biocontrol agents to reduce Vibrio sp., in shrimp culture practices.