Growth and Resistance to *Aeromonas hydrophila* of Indian Major Carp, Rohu (*Labeo rohita*) in Cisterns Treated with Sugarcane Bagasse as Artificial Substrate

K.M. RAJESH, K.M. SHANKAR, C.V. MOHAN and R.M. MRIDULA

Pathology and Biotechnology Laboratory
Department of Aquaculture, College of Fisheries
Karnataka Veterinary Animal and Fisheries Sciences University
Mangalore, India 575002

ABSTRACT

Biofilm of *Aeromonas hydrophila* developed *in vitro* when used as an oral vaccine gave a higher antibody titre and protection in carps compared with the free cells. The present study evaluated the effect of a biofilm developed on artificial substrates in carp ponds and their resistance to *A. hydrophila* infection. Fingerlings of rohu, *Labeo rohita,* were reared for 98 days in three treatments, namely: (i) sugarcane bagasse + cattle dung (SCD), (ii) sugarcane bagasse (S) and (iii) cattle dung (CD), on an equal dry weight basis. At the end of the 98 day period the specific growth rate of rohu was significantly higher ($P<0.05$) in SCD compared with CD and S groups. However, there was no significant difference ($P<0.05$) in the average survival between the 3 treatments. Rohu reared in the biofilm promoted ponds (SCD and S) had significantly higher antibody titres and protection upon challenge against *A. hydrophila* compared with those from cisterns treated alone with cattle dung. Promotion of biofilm had dual advantage in increasing fish growth and resistance against *A. hydrophila*.

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Corresponding author: K.M.Shankar, kalkulishankar@gmail.com

INTRODUCTION

Heterotrophs are the essential component of the food web, as these organisms decompose organic matter and release nutrients for algae and are also consumed directly by fish and fish food organisms (Colman and Edwards, 1987; Moriarty, 1997). Furthermore, they are not dependent on light and their nitrogen uptake potential to produce protein is higher than algae (Avnimelech *et al.,* 1986, 1992). Therefore, there is great scope for promotion and exploitation of heterotrophs for increasing aquaculture production. However, natural exploitation of heterotrophs by fish and fish food organisms is difficult and not economical, because of their small size which is a limiting factor. Exploiting the potential of heterotrophs through promotion of biofilm immobilized on artificial substrates was demonstrated as a strategy for boosting aquaculture production by Shankar *et al.* (1998). The potential of microbial biofilm on artificial substrate to increase fish production was later confirmed by several investigators (Ramesh *et al.,* 1999; Umesh *et al.,* 1999; Joice, *et al.,* 2002; Mridula *et al.,* 2003). In these studies, microbial biofilm promoted on artificial substrate such as sugarcane bagasse, paddy straw and *Eichhornea* leaves could boost fish growth by 50% compared with substrate free control. Furthermore, employing artificial substrates such as bamboo increased the growth of rohu by 77% (Wahab *et al.*, 1999) and growth of mahseer, *Tor putitora,* by 42% (Keshavanath *et al.,* 2001). Higher yield of planktophagic fish have also been recorded in traditional fishing methods such as acadja fisheries of West Africa (Welcome, 1972), brush parks of Sri Lanka (Sena Nayake, 1981), artificial reefs of the Philippines (Waltermath and Schirm, 1995) and floating islands of Loktak in India (Suresh, 1999).

Growth performance of *Oreochromis mossambicus* (Huchette *et al.,* 2000), *Catla catla, Labeo rohita, Cirrhinus mrigala* and *Cyprinus carpio* (Ramesh *et. al.,* 1999; Umesh *et al.,* 1999), *Labeo calbasu* (Wahab *et al.,* 1999), mahseer (Keshavanath *et al.,* 2001) and *Labeo fimbriatus* (Mridula *et al.,* 2003) have been evaluated with different substrates. Among these, in general browsers with fringed lips such as rohu, calbasu and tilapia grew better than others and the production was 50 to 77% higher in substrate-based culture systems compared with control systems.

Several observations, as well as controlled experiments, have indicated that heterotrophic production could also act as antagonists against pathogens in aquaculture systems (Avnimelech and Ritoo, 2001). Interestingly, in our preliminary studies, significantly higher serum agglutinating antibody titres and protection against *Aeromonas hydrophila* was recorded in common carp fry grown in ponds promoted with biofilm on sugarcane bagasse compared with the control ponds (Joice *et al.,* 2002). Earlier in our studies, biofilm of *A. hydrophila* developed *in vitro* and fed to Indian Major Carps (IMCs) as an oral vaccine through their feed induced significantly higher humoral and protective responses compared with the free-cell vaccine (Azad *et al.,* 1999a, 1999b and 2000). In biofilm mode, a bacterial pathogen occurring in several layers act as a biocapsule, which can withstand foregut destruction and can reach immune responsive hindgut to give better response and protection. The bacterium *A. hydrophila* is found ubiquitously in the aquatic environment and is regarded as an opportunistic pathogen frequently associated with haemorrhagic septicemia in carps, eels, milkfish, channel catfish, tilapia and ayu (Roberts *et al.,* 1992; Kuge *et al.,* 1992; Thune *et al.,* 1993; Leung *et al.,* 1995; Angka *et al.,* 1995).

Higher serum antibody agglutination titres and protection against *A. hydrophila* was observed in common carp reared from spawn to fry in a biofilm promoted system (Joice *et al*., 2002). These findings encouraged us to initiate further detailed study on growth and resistance in rohu - a column feeder with browsing habitat considered as appropriate model for biofilm exploitation in cisterns treated with sugarcane bagasse, with or without cattle dung.

MATERIALS AND METHODS

The experiment was carried out for 98 days in nine cement cisterns (5×5×1m) each with 15 cm soil base and filled with water to 80 ± 2 cm. Among the three treatments, treatment SCD received 0.8 unit (4 kg) of sugarcane bagasse and 0.2 unit (5 kg) of cattle dung, treatment S received 1.0 unit (5 kg) of sugarcane bagasse and treatment CD received 1.0 unit (25 kg) of cattle dung on dry weight basis. Cattle dung was procured from a local farm and sugarcane bagasse from juice extraction centers. The dosage of sugarcane bagasse and cattle dung was fixed based on dry weight of cattle dung, which is usually applied in fish culture ponds at 10,000 kg wet weight/ha. Sugarcane bagasse tied in 2-3 feet long bundles were suspended in the water column from horizontal bamboo beams. Fresh cattle dung was applied in the form of slurry.

Fingerlings of rohu, *Labeo rohita* (average weight of 2.08 g), procured from the government fish farm, B.R. Project, Karnataka State, were stocked at a density of 25/cistern (10000/ ha.), one week after the addition of bagasse and cattle dung.

Water parameters

Water was analysed once every three days for a number of different parameters. Temperature and pH were recorded using a Horiba water quality analyzer (Model U-10). Dissolved oxygen, total ammonia, nitrite and nitrate were measured following standard procedures (APHA, 1995).

Microbial enumeration

Total plate count (TPC) of bacteria in water and on sugarcane bagasse was estimated weekly on nutrient agar by the spread plate method (Ramesh *et al.,* 1999; Umesh *et al*., 1999). *A. hydrophila* was enumerated weekly by spread plate technique using *Aeromonas* isolation medium consisting of *Aeromonas* selective supplement (Hi Media, Bombay). *Nitrosomonas* sp*.,* and *Nitrobacter* sp*.,* in water and on substrate were enumerated weekly by the method of Rodina (1972). For estimation of TPC of bacteria, *A. hydrophila* and nitrifying bacteria on substrate, a known weight of bagasse was taken in a test tube containing physiological saline and bacterial cells in biofilm dislodged by vortexing for 3 mins. The cells were enumerated after culture and expressed as No/g.

Fish growth and survival

At least 50% of the stocked fish were collected and their individual length and total weight recorded at 15 days interval. However, on termination, at the end of 90 days, length and weight of all the surviving fish were recorded. Fish growth data obtained was subjected to ANOVA and Duncan's multiple range tests at $P < 0.05$ (Duncan, 1955; Snedecor and Cochran, 1968).

Evaluation of immune response and protection against *A. hydrophila*

Ninety days post-rearing of the fish, the substrate was removed in SCD and S and water exchanged fully in all the 3 treatments. Fishes were reared in these tanks for further 45 days on an artificial diet at 5% body weight. During this rearing period blood was drawn from five fishes in each treatment at an interval of 15 days and antibody titre estimated by agglutination assay (Sundick and Rose, 1980), and titre expressed as $\log_2$ values based on visual observations. The bacterium *A. hydrophila* (isolate AAh 2/96) was recovered from naturally infected *Clarius batrachus* and was obtained from the Central Institute of Freshwater Aquaculture, Bhubaneswar, India and used for the agglutination assay.

After 30 days of rearing in ponds providing artificial diet, twelve fishes from each treatment were transferred to 50L fiber-reinforced plastic tubs and each fish challenged with *A. hydrophila* (AAh 2/96). The bacterial isolate (*A. hydrophila*) was grown for 24 hrs in Tryptone Soya Broth (TSB) and used for challenge experiments. Bacterial growth from nutrient agar slants were harvested into 10 ml of 0.01M sterile phosphate buffered saline (PBS), centrifuged at 4,000 rpm for 20 min and the cell pellet resuspended in 10ml of 0.01M sterile PBS. After washing a further 3 times with sterile PBS, the bacterial concentration was determined using a spectrophotometer at 575nm and the cell density adjusted to 10^8 cell/ml. The dosage was determined by LD_{50} of the bacterium by injecting different doses of *A. hydrophila*. Fish from different treatment groups were administered with 0.1 ml of the pathogen (10^8 cells/ml) suspension by intramuscular injection. Fishes were observed for a week and external signs and mortality rates were recorded. Specific mortality of fish was confirmed by the re-isolation of the pathogen from the fish. Relative percentage survival (RPS) of fish was determined according to Amend, (1981) using the following formula:

$$RPS = 1 - \frac{\text{Percentage mortality in treatment}}{\text{Percentage mortality in control}} \times 100$$

RESULTS

Growth and survival

Significant difference ($P<0.05$) in average growth of rohu between the treatments was observed (Table 1). The growth was highest in SCD (38.97 g), followed by CD (31.93 g) and S (18.46 g). The overall survival of the fish was highest in the SCD (91.33%), followed by S (85.33%) and CD (84.00%). However, there was no significant difference ($P<0.05$) in the average survival of rohu between the treatments.

Table 1. Growth, survival and specific growth rate of *Labeo rohita.*

Parameter	Treatments		
	SCD	S	CD
Av. initial weight (g)	2.08 [a]	2.08 [a]	2.08 [a]
Av. final weight (g)	38.97 [a]	18.46 [b]	31.93 [c]
SGR (%/day)	1.41 [a]	1.06 [b]	1.32 [c]
Survival (%)	91.33 [a]	85.33 [a]	84.00 [a]

* Values with the same superscript in each row are not significantly different ($P < 0.05$)
SCD – Cisterns with sugarcane bagasse and cattle dung
S - Cisterns with sugarcane bagasse alone
CD - Cisterns with cattle dung alone

Microbial count

TPC of bacteria

In Table 2, details of microbial enumeration, total plate count (TPC) of bacteria *A. hydrophila* and nitrifying bacteria are given. Following the addition of cattle dung and bagasse, TPC of bacteria in water increased rapidly reaching a peak on day 15 in all the treatments (Figure 1a). Mean TPC ($\times 10^4$/ml) was significantly higher in SCD (13.51) followed by S (11.41) and CD (10.47). TPC of bacteria on bagasse also reached a peak on day 15, following which there was a gradual decrease (Figure 1b). The mean TPC of bacteria on bagasse ($\times 10^6$/g) was significantly higher in SCD (19.14) than in S (9.85). Overall, bacterial number on bagasse per unit weight was 100 times higher than that in water.

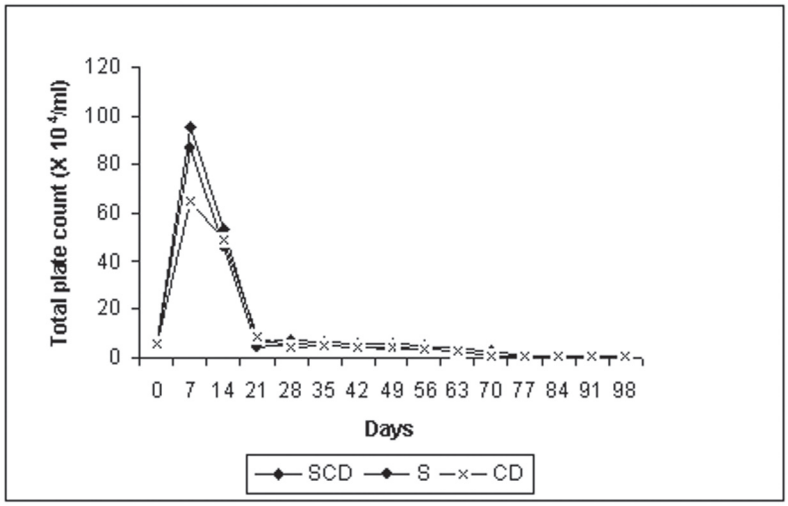
Aeromonas hydrophila count

There was a gradual increase in the *A. hydrophila* count in water after the addition of bagasse and cattle dung in S and SCD groups compared with CD. However, this was not statistically significant (Table 2). The peak was observed on day 21 in water and on bagasse in all the treatments following which there was a gradual decline. The mean *A. hydrophila* count in water ($\times 10^2$/ml) was almost similar in all the treatments with slightly higher values recorded in SCD (0.55) followed by S (0.45) and CD (0.40) (Table 2). The *A. hydrophila* count on bagasse ($\times 10^4$/g) was significantly higher in SCD (4.79) than in S (3.48).

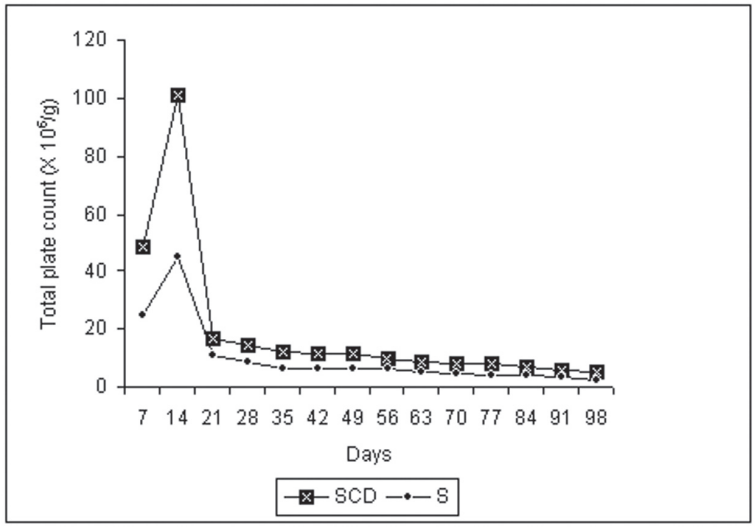
Water parameters

The mean values of water parameters are presented in Table 3. Following the addition of bagasse and cattle dung, dissolved oxygen dropped sharply and it was below 2.0 mg/l in all the treatments during the first week. However, during the subsequent weeks there was marked improvement in the dissolved oxygen. Mean dissolved oxygen (mg/l) was

(a)



(b)



SCD – Cisterns with sugarcane bagasse and cattle dung

S - Cisterns with sugarcane bagasse alone

CD - Cisterns with cattle dung alone

Figure 1. Total plate count of bacteria in (a) water and (b) on substrate in different treatments.

Table 2. Mean values of total plate count (TPC) of bacteria, *Nitrosomonas* sp., *Nitrobacter* sp. and *Aeromonas hydrophila.*

Parameter	Treatments		
	SCD	S	CD
TPC of bacteria in water ($\times 10^4 ml^{-1}$)	13.51 [a] (0.60-123.0)	11.41 [b] (0.38-59.0)	10.47 [c] (0.50-63.0)
TPC of bacteria on substrate ($\times 10^6 g^{-1}$)	19.14 [a] (4.0-127.0)	9.85 [b] (0.09-51.0)	-
Nitrosomonas sp. in water ($\times 10^2 ml^{-1}$)	1.75 [a] (0.0-5.0)	1.31 [a] (0.0-4.0)	1.80 [a] (0.0-5.0)
Nitrosomonas sp. on substrate (No.$\times 10^3$ g^{-1})	9.79 [a] (2.00-23.0)	7.95 [b] (1.0-19.0)	-
Nitrobacter sp. in water (No.$\times 10^2$ ml^{-1})	2.22 [a] (0.0-6.0)	1.82 [a] (0.0-3.0)	1.99 [a] (0.0-4.0)
Nitrobacter sp. on substrate (No.$\times 10^3$ g^{-1})	11.95 [a] (2.0-27.0)	8.17 [b] (2.0-19.0)	-
Aeromonas hydrophila in water (No.$\times 10^2$ ml^{-1})	0.55 [a] (0.10-1.54)	0.45 [a] (0.10-1.35)	0.40 [a] (0.06-1.32)
Aeromonas hydrophila on substrate (No.$\times 10^4$ g^{-1})	4.79 [a] (0.14-27.20)	3.48 [b] (0.06-21.0)	-

* Values are means of three tanks and fifteen sampling days (N = 45) for water and three tanks and fourteen sampling dates for substrates (N = 42).

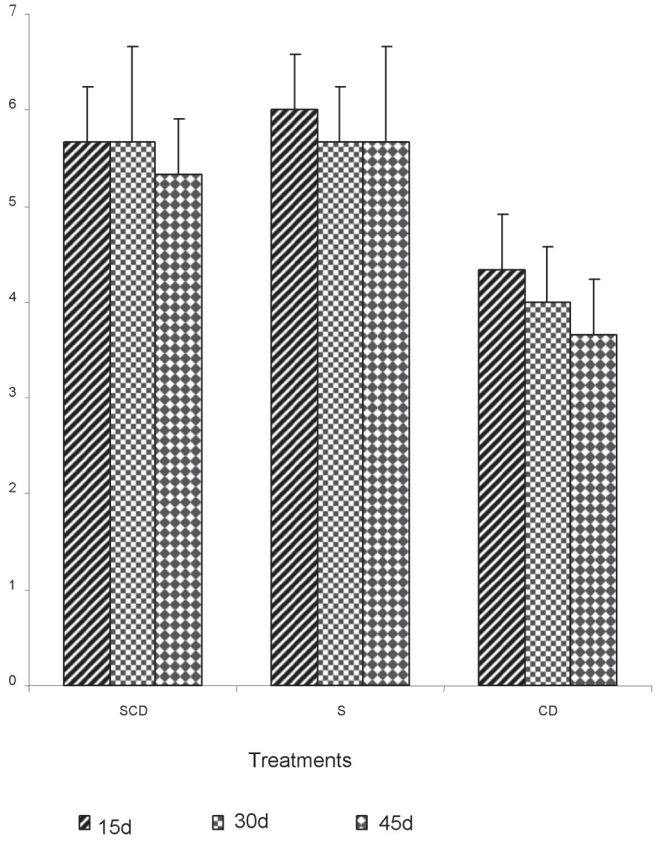
** Values in parenthesis indicate range

*** Values with the same superscript in each row are not significantly different ($P < 0.05$)

SCD – Cisterns with sugarcane bagasse and cattle dung

S - Cisterns with sugarcane bagasse alone

CD - Cisterns with cattle dung alone



SCD – Cisterns with sugarcane bagasse and cattle dung

S - Cisterns with sugarcane bagasse alone

CD - Cisterns with cattle dung alone

Figure 2. Serum agglutination titer at 15, 30 and 45 days after 90 days of rearing in biofilm promoted tanks in different treatments.

significantly higher ($p<0.05$) in SCD (8.06) than that in CD (7.90) and S (7.72). The mean total ammonia (µg at./l) was significantly lower in SCD (3.49) and S (3.99) than that in CD (5.07), while the mean nitrate-nitrogen (µg at./l) was significantly higher in SCD (2.19) compared with S (1.80) and CD (1.80).

Immune response and protection in *Labeo rohita* against *A. hydrophila*

Antibody titre and protection

Mean antibody titre ($-\log_2$) in the fish was significantly higher in SCD (5.55) and S (5.77) than that of CD (3.99). A reduction in the antibody titre was recorded 15 days after harvest in all the treatments which was more pronounced in CD (Figure 2).

Relative percentage survival (RPS)

The mortality rate was 8.33% in SCD, 0% in S and 75% in CD after challenging with *A. hydrophila,* AAh 2/96 (Table 4). Relative percentage survival was higher in S (100) than that of SCD (88.89).

Table 3. Mean values of water quality parameters.

Parameter	Treatments		
	SCD	S	CD
Water temperature (°C)	28.20 [a] (24.2-31.5)	28.00 [a] (24.0-31.3)	28.40 [a] (24.4-31.2)
pH	7.80 [a] (7.06-8.22)	7.60 [a] (7.02-8.23)	7.80 [a] (7.02-8.27)
Dissolved oxygen (mg/l)	8.06 [a] (2.28-9.13)	7.72 [a] (2.28-9.13)	7.90 [a] (1.37-9.58)
Total ammonia (µg at./l)	3.49 [a] (2.09-7.35)	3.99 [a] (2.50-6.75)	5.07 [b] (2.48-9.68)
Nitrite-nitrogen (µg at./l)	1.29 [a] (0.44-2.04)	1.36 [a] (0.60-2.16)	1.81 [b] (0.78-2.96)
Nitrate-nitrogen (µg at./l)	2.19 [a] (0.54-5.24)	1.89 [b] (0.54-4.24)	1.80 [b] (0.54-4.04)

* Values are means of three tanks and thirty-four sampling days (N = 102).
** Values in parenthesis indicate range
*** Values with the same superscript in each row are significantly different (P>0.05)

SCD – Cisterns with sugarcane bagasse and cattle dung
S - Cisterns with sugarcane bagasse alone
CD - Cisterns with cattle dung alone

Table 4. Relative percentage survival in different treatments after challenging against *A. hydrophila*.

Treatments	No. of fish challenged	No. of fish survived	Percentage survival (%)	Relative percentage survival (RPS)
SCD	12	11	91.66	88.89
S	12	12	100	100
CD	12	3	25	-

SCD – Cisterns with sugarcane bagasse and cattle dung
S - Cisterns with sugarcane bagasse alone
CD - Cisterns with cattle dung alone

DISCUSSION

Growth of rohu was highest in SCD followed by CD and S and there was significant difference in growth between them. However, there was no significant difference in survival of fish between the treatments. Better growth and survival of rohu and other IMCs with substrate supplemented with manure compared with manure or substrate alone has been reported by us and other authors (Ramesh *et al.,* 1999; Umesh *et al.,* 1999; Joice, *et al.,* 2002; Mridula *et al.,* 2003). The TPC of bacteria in the water was higher in SCD compared with S and CD treatment groups. It is interesting that CD had a lower total plate count than the group in the S treatment, which could be due to a higher density of TPC on the S substrate. Total bacterial plate counts on this substrate was 100 fold higher than those calculated for the water per unit. However, there was significant difference in TPC between SCD and S. Therefore, overall, it is total higher TPC on substrate and in water in the SCD than that in S or CD on which rohu could browse. This may be the reason for the enhanced growth and survival in these treatment groups. Rohu is a column browser (Das and Moitra, 1955; Dewan *et al.,* 1991) and is known to thrive well by browsing on substrates (NFEP, 1997). Plankton production was not enumerated because our earlier studies have demonstrated clearly that higher TPC on substrate favour higher plankton production (Ramesh *et al.,* 1999; Umesh *et al.,* 1999; Joice, *et al.,* 2002; Mridula *et al.,* 2003). Better growth and survival in SCD compared with CD or S could also be due to lower ammonia and nitrite in the former treatment. Such low level of ammonia and nitrite have been observed in substrate added ponds (Langis *et al.,* 1988; Ramesh *et al.,* 1999; Umesh *et al.,* 1999; Joice, *et al.,* 2002; Mridula *et al.,* 2003). Additional growth of nitrifying bacteria on the substrate could have acted as an *in situ* biofilter.

Antibody titre against *A. hydrophila* in rohu was highest in SCD followed by S and CD. Little difference was detected in the antibody titres between the SCD and S-treatment groups compared with the wide difference between SCD and CD or S and CD. Interestingly, growth pattern was also different being higher in SCD followed by CD and S. The *A. hydrophila* count in the water was slightly higher in the SCD group followed by S and CD. However, A. *hydrophila* count on the substrates was 100 fold higher in the SCD than in S. The data generated from this study would suggest that the higher *A. hydrophila* concentration on the substrate has favoured a higher antibody titre in the SCD group.

Biofilm of *A. hydrophila* on the substrate could have acted as *in situ* immunomodulator such as a bacterin, in rohu when exposed to the pathogen. Oral vaccination of carps with biofilm of *A. hydrophila* incorporated in feed has given better antibody titre and protection compared with free cell (Azad *et al*., 1999a and 1999b). Furthermore, it was interesting to note that the antibody titre dropped more rapidly after 15 days in CD compared with that in SCD and S. This could be due to retention of *A. hydrophila* biofilm antigen in immune response sites of the fish for a longer duration compared with that of free cell antigens in CD treated groups. Longer retention of biofilm antigen in immune responsive sites with higher antibody titre in carps has been demonstrated by antigen localization with monoclonal antibody in immunohistochemistry (Azad *et al*., 2000).

The relative percentage survival of the experimental groups was higher in those given SCD and S treatments which corresponded with a higher antibody titre in both these group. In contrast, a very poor survival was noticed in the CD fish group in which the antibody titre was low and was decreasing rapidly. This supports the hypothesis that there was an immunomodulation in the SCD and S groups leading to an enhanced protection against bacterial challenge. This also clearly shows the importance of biofilm on substrate to boost resistance against opportunistic bacterial pathogens found ubiquitously in the aqueous environment such as *A. hydrophila*. These findings support our earlier observations of higher antibody titre and RPS recorded in common carp spawn reared in substrate-based ponds compared with control (Joice *et al*., 2002). Several observations as well as controlled experiments have indicated that heterotrophic population in pond act as antagonists against pathogens (Avnimelech and Ritoo, 2001).

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