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INFLUENCE OF BIOREMEDIATION ON THE GROWTH AND SURVIVAL OF *CYPRINUS CARPIO* VAR *KOI* USING AQUACULTURE WASTE WATER

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ABSTRACT

A number of commercial products including live bacterial inocula, enzyme preparation are being promoted for use in improving water quality in aquaculture systems. The present study is an attempt to know the effect of using mixed microbial consortia in the form of a commercial probiotic (*Bacillus subtilis, Nitrosomonas, Nitrobacter* and *Rhodobacter*) at various concentration of 0.25g/m^3 (T-1), 0.5 g/m^3 (T-2), 1 g/m^3 (T-3) and control (without probiotics)to know its ability to remove the nitrogenous compounds, Phosphate-P, BOD and COD from aquaculture waste and making it fit for rearing of *Cyprinus carpio* var *koi*. The initial physico-chemical parameters of aquaculture waste water collected from the farm revealed that it was not fit for rearing of fishes. Laboratory study of the microbial inoculum applied demonstrated a greater rate of removal of nitrogenous compounds, phosphate-P, BOD and COD. The total heterotrophic count showed a gradual increase in its count during the bioremediation of the aquaculture waste water when compared to that of control (without probiotics). Probiotics added at the rate of 1g/m^3 showed better remediation results when compared to other dosages. The bioremediated rearing system also showed improved water quality as probiotics were added at regular interval .The growth parameters of koi carps in the bioremediated system showed better survival rate (97.25%), average length gain (65.1 ± 1.159 mm), average weight gain (5.279 ± 0.128 g)and specific growth rate (3.37) in treatment -3(1g/m^3) than other treatments and control. The study proved that the probiotics used was instrumental in improving the water quality, growth rate and survival rate of koi carps. The novel thought behind this study is that aquaculture waste water instead of discharging into the open system can be reused again after bioremediation.

INTRODUCTION

Aquaculture effluent when discharged into the neighbouring water body, add high levels of BOD, inorganic particulate matter, nutrients in the form of ammonia, nitrite, nitrate, phosphate, sulphides and lipids that impact the environment. At present the two major challenges confronting aquaculture are sustain-

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able usage of resources with economic growth and environmental management which can be achieved by responsible use of natural resources through conservation and sustainable practices. There are various physical and chemical methods available for treating aquaculture effluent. All these methods do help in treating the effluent but are mostly in terms of capital investment, energy consumption and maintenance requirement, and very little research has been focused on aquaculture waste water effluent. The development of an effective, low-cost treatment is imperative if aquaculture has to expand continually. The newest attempt being made to improve water quality in aquaculture effluent is the application of probiotics and /enzymes. This type of technique is known as "bioremediation" which involves use of microorganisms in ponds to enhance mineralization of organic matter and mitigate undesirable waste compounds. It has emerged as a sustainable strategy focused on alleviating the negative effect of aquaculture effluent. The rapid growth of microbes, their existence in diverse environments and their nutritional requirements are considered as useful characteristics for their use as bioremediators. In aquaculture, their role is enhanced by maintaining balanced bacterial population in various ways like enriching of larval food, inclusion in the diet, or addition to the water as a remediating agent. Once nitrogenous nutrients are released in the environment, the nitrification process takes place which is performed by nitrifying bacteria such as *Nitrosomonas* and *Nitrobacter*. However, the environment receiving aquaculture effluent have limited capacity to process and incorporate those nutrients into food chain and biogeochemical cycles, which means that considerable amount of nutrients tend to accumulate, causing ecological imbalance. This is commonly known Probiotics, mineralization, sustainable, consortium, hypernitrification, specific growth rateas hypernitrification (Boyd, 2003). Bioremediation is considered as a safer, cleaner, cost effective, environmental friendly technology for treating effluents having wide range of pollutants. However, the bioremediation process is relatively slow. The bacteria begin to accelerate the process of degrading the contaminants by using them as an energy source, ultimately cleansing the environment (Lopes *et al*., 2011). This natural process is slow compared to the man-assisted program to speed up the environmental bioremediation. In recent years, research on probiotics for aquatic animals is increasing with the demand for environment-friendly aquaculture practices (Angélica *et al*., 2014; Natesan *et al*., 2014; Rajinikanth *et al*., 2010; Raja *et al*., 2014; Wang *et al*., 2005). Ornamental fish farming is an important primary industry as it plays an important role in the socio-economic upliftment of backward class and women in our country. *Cyprinus carpio* var koi (Linnaeus, 1758) member of the family Cyprinidae, is characterized by a wide diversity of colors and color patterns and is an ornamental variety of common carp. Koi are considered "hardy" fish because they can survive in poor water quality similar to that of common carp. They are cold water fish, but benefit from being kept in 15-25°C range and do not react well to long cold winter temperatures. It can with stand sudden changes in the water quality during transportation and shifting and they are fast growers and well adapted to have commercial feed.

Not much attempt has been made so far to study the efficacy of probiotics in treating aquaculture effluent and rearing of ornamental fish in it. Hence, the present study was aimed at investigating the influence of probiotics on reducing the organic and nitrogenous load from the effluent and making it fit for rearing and comparing the growth and survival with control which is fresh water thereby reducing the pressure on the usage of precious water resource

MATERIALS AND METHODS

Collection of aquaculture waste water and survival test for koi carps

Aquaculture waste water was collected from M/s Sidha aqua farm, Sawyerpuram, located 15 kms south west of Thoothukudi and transported to the college campus. Small troughs of 50 liters capacity were taken and the effluent was diluted at 0%, 25%, 50% and 75% with fresh water. The effluent was continuously aerated using a high blow air pump. 10 numbers of koi carp young ones (average length of 25 ± 0.5 mm and average weight of 1.2 ± 0.29 g) were introduced into each trough and the survival and mortality rate was recorded. The initial water quality parameters and nutrient content were analyzed and experiment was conducted for a period of five days. The number of fishes survived was assessed to justify the need for treating the aquaculture effluent. Hundred percent mortality of koi carp was observed at the end of 3rd day in 0% diluted effluent. From this it was inferred that without bioremediation the waste water effluent could not be used for rearing of fish.

Bioremediation of aquaculture waste water in culture tanks

Samples for nutrient analysis were collected in separate plastic sample containers in the farm. The physico-chemical characteristic of the effluent water viz., temperature, pH, Dissolved oxygen, BOD, COD, alkalinity, hardness and nutrients such as total ammonia nitrogen (TAN), nitrite-N, nitrate-N and phosphate were determined following the standard protocols from APHA (2012). These parameters were determined before the addition of probiotics.

Probiotic application in experiment tanks

The probiotics, a mixed consortium of bacterial strain such as *Nitrosomonas* sp, *Nitrobacter* sp, *Bacillus subtilis* and *Rhodobacter* sp at the rate of 0.25g, 0.5 g, 1 g/m^3 were activated by adding them in 500 mL of water sample and left as such with continuous aeration for 2 hours. Then the probiotics were applied in the treatment tanks uniformly.

In real time culture tanks

Samples were collected and the water quality parameters were studied using the standard procedure (APHA 2012). The mixed consortia was added at the rate of 0.25, 0.50 and 1.0 g/m^3 to study the bioremediation process and reduction of the nitrogenous compounds to the acceptable limit.

Bacteriological analysis

Bacteriological analyses were carried out for the enumeration of THB (total heterotrophic bacteria) using nutrient agar. Column water samples were collected and THB was enumerated by adopting the spread plate method. All the determinants were carried out in duplicates. Following incubation, plates containing viable colonies >30 and <300 were used to calculate the bacterial population. The colonies were counted and expressed as cfu/mL.

Chlorination

After the bioremediation of waste water chlorination was done by adding calcium hypochlorite (Bleach 30%) at the rate of 20 ppm (Ponnusamy, 1997) in order to prepare the culture water for rearing experiment.

Experimental fishes

Koi carp having an average length of 25 ± 0.5 mm and average weight of 1.2 ± 0.29 g were acclimatized for 10 days under laboratory condition in FRP tanks containing well aerated water. The feeding of the test animals was stopped before two days of the commencement of the culture experiment. The rearing experiment was conducted for a period of 105 days.

Rearing experiment

The *Koi* carps were stocked in the bioremediated experimental tanks at the rate of 1 fish/10 litres. (Jha and Bharat, 2014). Probiotics were added in three bioremediated treatment tanks at the rate of 0.25 g/m^3 (T-1), 0.50 g/m^3 (T-2)and 1.0 g/m^3 (T-3). One tank was maintained as Control tank where only fresh water was used for the rearing of koi carp without any probiotic treatment. Routine water quality parameters were determined weekly twice for a period of 105 days. The koi carps were fed twice a day at the rate of 5 % of their body weight with a commercial fish feed having a crude protein content of 32%.

Growth sampling was done every fortnight and the length in mm and weight in g were recorded for a period of 105 days of culture. From the pooled growth data of koi carp, length and weight gain, mean length and mean weight gain, and specific growth rate (SGR) were estimated. The results of the study were analyzed statistically using ANOVA and the level of significance at $P<0.05$ and $P<0.01$ were considered for validation (Snecdecor and Cochran, 1967).

RESULTS AND DISCUSSION

Physico-chemical parameters of raw waste water

The water quality parameters of raw aquaculture waste water before and after bioremediation in T-1, T-2,T-3 and control are presented in Table 1. The total heterotrophic count in the treatment tanks and control before and after application of probiotics are given in Table 2. Growth parameters of koi carp in the control and bioremediated treatment tanks are given in Table 3. Water temperature in all the tanks were at the range of 28-30°C since it was an indoor set up there was no much swing in the temperature. pH in the treatment tanks were slightly less than the control during bioremediation and this might have been due to the enhanced microbial activity leading to greater levels of respiration and increase in carbon-di-oxide. The initial TAN value of raw aquaculture waste water was 0.916 mg/L higher than the permissible limit of 0.1 mg/L (Boyd and Gross, 1998). Level of TAN cannot be removed easily however it can be converted to non-toxic nitrate by nitrifying bacteria.

The dosage of probiotics added at the rate of 0.25

g/m3, 0.5 g/m^3 and 1g/ m^3 had different levels of efficiency in terms of removal of nutrients.The rate of removal of TAN was higher in T-3 when compared to other treatments and control. At the end of bioremediation there was 91% removal of TAN in T-3. The total heterotrophic bacterial (THB) count in control, T-1,T-2, and T-3 (Table 2) showed that the probiotics was well estabalished in the system on the 7th day of bioremediation (2.81 x 10^9 cfu/mL). The heterotrophic bacteria are known to utilize nitrogen rich substances and release ammonia or ammonium salts (Padmavathy *et al.*, 2012). Also THB such as *Bacillus* also exhibits a synergistic effect that will improve the nitrification process. In this case, such compensation by the *Bacillus* sp would have improved the nitrification by the nitrifying bacteria in the probiotics that made a check over the accumulation of TAN by converting it into nitrate-N via nitrite-N being an intermediate product. Nitrate concentration in T-3 was more due to oxidation of various forms of inorganic nitrogen.

A favourable range of 0.1 to 4.0mg/L as admissible was reported by Sahar (2014).The values of nitrate-N were within the safe limit. It took long duration for oxidation of TAN to nitrate-Nwhich could not be explained in the light of the available literature. The dissolved oxygen content is an index of water quality and the average dissolved oxygen values in the treatment tanks registered a gradual increase from 1.47 to 6.32mg/L in T-3. Dissolved oxygen in the beginning was low due to the high organic and nitrogenous load. In the present study the long duration for the complete bioremediation would have been due to slowing up of the nitrifying process due to the limited aeration supply as nitirfiers are strictly aerobic in nature. Phosphate-P is one of the major nutrients required for physiological process in living organisms. At the same time it can also be considered as a pollutant leading to eutrophic condition.

The study showed 81% reduction of phosphate in T-3 (0.71 mg/L). Bhatnagar *et al*. (2004) stated a safe level of 0.03-2 mg/L of phosphate-P as the acceptable range for rearing fishes. Aquaculture system deemed to be polluted and unsuitable if BOD and COD levels exceed 10 ppm and 20 ppm (Rath, 2011). BOD and COD reduction was 100% in T-3 at the end of the bioremediation period which showed that the

Table 1. Physico chemical characteristics of raw aquaculture waste water before bioremediation

S.No.	Parameters	Before bio-remediation	After bioremediation using probiotics and aeration			Only aeration Control
			T-1	T-2	T-3	
1.	Temperature	29°C	28°C	28°C	28°C	28°C
2.	pH	8.34±0.008	8.09±0.020	8.08±0.024	8.03±0.014	8.21±0.032
3.	Dissolved oxygen	1.08±0.003 mg/L	4.87±0.014	6.32±0.003	6.85±0.008	2.99±0.009
4.	Alkalinity	134.80±0.134 g/L	132.43±0.437	132.19±0.389	132.21±0.291	133.31±0.221
5.	Hardness	125.60±0.251 mg/L	122.89±0.344	122.81±0.456	122.31±0.324	123.09±0.516
6.	Total Ammonia Nitrogen	0.916±0.001 mg/L	0.497 ±0.0007	0.213 ± 0.0007	0.074 ± 0.0001	0.655±0.008
7.	Nitrite-N	0.0068 ± 0.0001 g/L	0.0048±0.00005	0.0034±0.00001	0.0016±0.00001	0.0055±0.0003
8.	Nitrate-N	0.066 ± 0.0001 mg/L	0.085 ± 0.0003	0.089 ± 0.0002	0.089 ± 0.0001	0.054 ± 0.0001
9.	Phosphate-P	3.85 ± 0.007 mg/L	2.32 ± 0.003	1.66 ± 0.013	0.71 ± 0.001	2.84 ± 0.001
10.	BOD	15.63 ± 0.033 mg/L	4.22 ± 0.014	2.24 ± 0.006	0.0±0.0	9.38 ± 0.011
11.	COD	42.63 ± 0.085 mg/L	8.74 ± 0.066	6.28 ± 0.023	0.0±0.0	15.66 ± 0.062

Table 2. Total heterotrophic bacterial count in Treatment tanks and control

Duration of bacterial analysis	CONTROL	T-1	T-2	T-3
Before application	2.6 x 10^3	1.23 x 10^4	1.91 x 10^4	2.93 x 10^6
After 24 hours	1.2 x 10^4	1.32 x 10^4	2.89 x 10^5	2.88 x 10^7
4th day	6.4 x 10^3	2.13 x 10^4	2.50 x 10^7	2.90 x 10^7
7th day	3.0 x 10^3	2.72 x 10^5	2.59 x 10^7	2.81 x 10^9

Table 3. Growth parameters of koi carp in the bioremediated treatment tanks and control tank

Growth parameters	Control		T-1		T-2		T-3	
	Initial sampling	Final sampling	Initial sampling	Final sampling	Initial sampling	Final sampling	Initial sampling	Final sampling
Total length (mm)	35.6±1.01	44.8±0.628	38.2±0.940	53.6±1.55	38.7±0.789	55.0±2.442	42.1±1.251	64.1±1.159
Total body weight (g)	2.38±0.057	2.89±0.162	2.449±0.053	4.11±0.194	2.483±0.036	4.316±0.303	2.786±0.908	5.279±0.128
Length gain (mm)	6.8±0.013	0.9±0.001	10.0±0.02	2.5±0.0075	9.1±0.018	2.3±0.002	3.9±0.007	2.82±0.002
Weight gain (g)	0.567±0.001	0.091±0.003	0.637±0.001	0.235±0.705	0.622±0.001	0.274±0.004	0.803±0.001	0.108±0.0001
Mean length gain (mm)	0.454±0.009	0.66±0.0001	0.67±0.134	0.16±0.0032	0.61±0.001	0.153±0.0003	0.26±0.026	0.188±0.0003
Mean weight gain (g)	0.0378±0.007	0.06±0.0001	0.042±0.0008	0.015±0.0003	0.041±0.008	0.018±0.002	0.053±0.0005	0.007±0.0002
SGR (% body weight gain/day)	1.79±0.005	2.1±0.101	2.06±0.005	0.78±0.013	1.92±0.002	0.80±0.001	3.37±0.007	1.09±0.011

probiotic bacteria were able to oxidize the organic compound in the waste water more efficiently. The varations for pH, dissolved oxygen, alkalinity, hardness,TAN, nitrate-N, Phosphate-P, BOD and COD were statistically significant below 0.01 ($P<0.01$), between control and treatment tanks.

Culture of *Cyprinus carpio* var *koi* was carried out in three treatments viz, T-1, T-2 and T-3 (probiotics applied at the rate of 0.25 g/m^3, 0.5 g/ m^3 and 1 g/ m^3) and control to study the growth and survival. In all bioremediated treatment tanks, the conservative parameters did not show much fluctuation when compared to that of control. All these parameters were within the permissible limit till the end of the culture period. The water quality characteristics determined during the culture period in the bioremediated rearing system showed a positive impact due to the addition of probiotics. The variation in the level of nutrients studied was statistically significant at ($P<0.01$) level between the control and bioremediated treatment tanks.

The use of beneficial microbes alters or regulates the composition of bacterial flora in the rearing system and koi carps showing an improved growth rate in terms of length and weight gain during the culture period in T-3 when compared to T-1, T-2 and control. A survival rate of 97.25% in bioremediated treatment tank-3 was obtained showing a positive influence of beneficial microbes in the health status of koi carps when compared to T-1 (91 %),T-2 (95 %) and control (90.5%).

The bioremediated system provided a conducive environment for the growth of koi carps. The stocking density was maintained at 1 fish/ 10 litres in order to avoid stress in the fish because the system is a bioremediated system. Although more literature is available on the use of probiotics as feed supplement, information on the use of probiotics directly into the rearing system is scanty. Similar study was conducted by Cha *et al*. (2013) in which probiotics was directly used as water additive. The growth performance was improved due to the presence of *Bacillus* sp in the probiotic consortia which are known to be better converters of organic matter than gram negative bacteria (Verchuere *et al*., 2000). These groups of microbes are able to colonize both in the culture water and in the digestive tract of the fish thereby increasing the survival rate. They produce group of enzymes such as protease, lipase that can help improve digestion and nutrient absorption, resulting in better use of food and animal growth. *Bacillus* sp are able to out compete other bacteria for nutrients and space and antagonistically exclude other bacteria through the production of antibiotics (Abraham and Banerjee, 2007).

Overall growth and specific growth rate in all the treatment tanks were higher when compared to control. However, the performance in treatment tank-3 had a higher profile for overall length gain, weight gain, specific growth rate, and survival rate. A similar profile of growth parameters such as length gain, weight gain and specific growth rate was recorded by Nihan *et al*. (2013) in *Cyprinus carpio*. The current study emphasize that probiotic if used in right dosage with the compatible species combination could prove to be an important management tool in removing the nitrogenous species from the waste water and making it fit for rearing purpose. Microorganisms being nature's original recycler can detoxify waste water, contaminated soils, etc., thus getting wide acceptance that "bioremediation" will now become a common terminology in waste water management. However, the efficiency depends on understanding the nature of the organism, their competition between species and their mode of action. This technology of rearing of *koi* carps can be simple, cost effective technology for farmers involved in ornamental fish culture.

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