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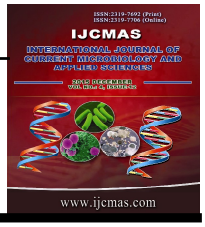

Original Research Article

Supplementation of *B. cereus* as Probiotic in Fish Feed of *Trichogaster Trichopterus* (Blue Gourami) and Calculating its Growth and Survival

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Gourami, Bacteriocin, 16s rRNA, Growth rate

A B S T R A C T

The study investigated the effect of *B. cereus* probiotic on the growth performance in *T. Trichopterus*. *B. cereus* strain isolated from raw milk was characterized by means of standard biochemical and 16S rRNA gene sequencing studies. Fish feed with and without bacteriocin was prepared and it was fed to the juvenile fishes. After 60 days trial, specific growth rate was increased with the addition of Bacteriocin in fish diets. Likewise the survival rate of juvenile *T. trichopterus* was 50% for control and 94% the fish diet supplemented with the bacteriocin produced by *B. cereus*. Thus it was found that in addition of being effective bacteriocin producing *B. cereus* could also promote the growth of the fish effectively and thus it play an important role in aquaculture nutrition.

Introduction

Farming of aquatic organisms like crustaceans, fish, molluscs and aquatic plants are termed as Aquaculture and it is also identified as aqua farming. Aquaculture is diverged from commercial fishing because the cultivation of fresh water and salt water populations under controlled conditions is former and harvesting of wild fish is later. (ASAP 2009). Ornamental fish are those small sized, live and colourful fish kept in home or public aquaria or in garden pools for recreation. Freshwater ornamental fish contribute 85% of the total global ornamental fish trade (Mohanta2011).

Trichogaster trichopterus (Pallas 1770; Rajesh et al. 2011) commonly called Blue gourami fish, is a common and popular fresh water aquarium fish belonging to the family Belontiidae. An ideal ornamental species should posses attributes like captive survival (including acceptance of artificial diet), attractive colouration pattern, exotically patterned (endemicity), hardiness, peaceful nature, compatible with other species and above all tiny size, so that they can be reared in aquarium throughout their life span. One of the major problems for the growth of ornamental fish farming is the non-

availability of species specific nutritionally balanced diets.

Probiotics are well known and routinely used additives in the main livestock species. They claim to improve gut health by stabilizing gut flora being their effect reflected in a better overall health status, welfare and performance of the animals. In aquaculture probiotics are administered by feed and/or as a water additive. The supplementation of probiotics through feed is a better method to ensuring the efficiency of the probiotic bacteria in the GI tract of the fish. However, their use in fish feed production is still scarce (EPA 2012).

This study was designed to evaluate the use of Bacteriocin produced by *Bacillus cereus* as a partial replacement for fishmeal protein in practical diets for blue gourami in terms of growth and survival.

Materials and Methods

Bacterial strains

Aseptically collected milk sample was serially diluted in 0.5% peptone water and it was inoculated by spreading on the Nutrient agar (Hi media, India). The inoculated plates were incubated at 37^0C for 48 h.

Screening of Bacteriocinogenic Strains

Pure culture of the isolate was prepared based on the colony morphology. The cell free supernatant was prepared from the 25 mL of nutrient broth inoculated with 24h old culture. (Coventry et al. 1996). Using that supernatant the antimicrobial activity was analysed against Fish pathogen *Vibrio vulnificus* (MTCC 1145) as indicator strain procured from MTCC, Chandigarh, India.

Identification of Bacteriocinogenic Strain

Further identification of the strain showed better activity than other isolates was subjected to 16S rRNA sequence analysis and this was performed at Macrogen, Korea.

Phylogenetic Analysis

Using BioEdit Sequence Alignment Editor (Version 7.1.9) the partial 16S rRNA sequence was assembled. Searches were done against the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) program. Based on the 16S rRNA gene sequence of the isolate phylogenetic tree was constructed using the neighbour-joining method by Mega 6.

Production and Partial Purification of Bacteriocin

To the 1000 mL of nutrient broth the isolated culture was inoculated and incubated at 37 °C in the orbital shaker with the rpm of 150 for 72 hours. Centrifuged the incubated broth at 10 000×*g* for 20 min and the supernatant was collected and partially purified by adding $(NH_4)_2SO_4$ at 70 % of saturation level, followed by dialysis for 12 h. Centrifuged at 20 000×*g* at 4 °C for 30 min to collect the pellet and dissolved in phosphate buffer (0.1 M, pH=7.0). The dissolved pellet can be stored at 4 °C for further use.

SDS-PAGE Analysis

The molecular weight of partially purified bacteriocin samples was determined by SDS-PAGE (Schagger and Von Jagow 1987). 16% separating gel, 10% spacer gel and 6% stacking gel was used with vertical slab gel electrophoretic apparatus. From the relative mobility of the standard molecular weight markers the molecular weight was calculated.

Characterization of Bacteriocin (Paik et al 1997)

Effect of pH

To 4.5 mL of nutrient broth at different pH values ranging from 3.0 to 11.0, with an increment of 1, 0.5 mL of partially purified bacteriocin was added and incubated for 30 min at 37°C. Each of the bacteriocin samples treated at different pH values was assayed against indicator bacteria by well diffusion method.

Effect of Temperature

To 4.5 mL of nutrient broth in the test tubes 0.5 mL of purified bacteriocin was added and it was treated for 10 min at different temperature, *i.e.* 40, 50, 60, 70, 80, 90, 100 and 121°C after overlaying with paraffin oil to prevent evaporation and the bacteriocin activity was measured by well diffusion assay.

Collection and Stocking Fingerlings

Fifty healthy and active juveniles of 40 days old *Trichogaster trichopterus* with an average wet weight of 2g ±0.2, and body length 2.5cm ±0.3 were collected from a local supplier and transported to the lab in plastic bags filled with oxygen. Fishes were acclimatized by placing the bags in a 200 litre plastic container until water temperature was equalized inside the bags at 27 $\pm 1^0$C (Axelrod et al. 1997). The container was supplied with tap water passed through a filter (5 µm diameter) and gentle aeration was provided.

Fish were divided in to four different groups and fed them with two different feeds. Group I was treated as control, fed with 38% protein diet and group II were fed with formulated diet with bacteriocin produced by the isolated bacterial isolate. Triplicates were maintained for each test diets. Sixty days experiment was conducted with a view of observing the effect of supplemented feeds on the growth and survival of the fish *T. trichopterus*.

The hydrobiological parameters such as Temperature, pH, Water Hardness and Dissolved oxygen were monitored. The tanks were drained twice a week to remove accumulated faeces from the bottom and replenished with freshwater.

Collection of Feed Ingredient

The low cost quality fish feeds of same protein level was prepared using some locally available ingredients such as fish meal, groundnut oil cake, soya meal, rice bran, tapioca powder and wheat flour.

Feed Preparation

Grounded and sieved the collected dietary ingredients. The composition of the dietary mixture was tabulated in the Table 1. Using hand operated pelletizer about 2 mm size feeds were prepared, sundried and stored in air tight container. The dietary preparation of 38% protein was done by the method of Hardy (1980).

Feeding

Fish were fed 4% of body weight twice a day during the 60 days experiment. Weighed feed was given in a feeding tray. One hour later the unconsumed feed was removed and dried in a hot air oven at 80^oC. For every 10 days feed consumption was estimated by subtracting the amount of unconsumed dry feed from the total dry weight of feed given. The feeding rate was computed as.

$$Feeding\ rate\ (mg\ g^{-1}\ live\ fish\ day^{-1}) = \frac{Amount\ of\ feed\ consumed(mg)}{\text{Initial wet weight of fish g x No. of days}}$$

Growth Estimations

Fish were weighed at the beginning of the experiment and on every 10 days. Growth or weight gain was calculated with the divergence between the initial wet weight on the day of sampling. Specific Growth Rate (SGR) was analysed by the difference between wet weight at the beginning of the experiment and on the day of sampling as

$$SGR\ (\%\ day^{-1}) = \frac{\ln Wt1 - lnWt0}{t1} x100$$

Where ln Wto and lnWt1 are the natural logarithm of initial and final weights of the fish during each sampling period and t1 is the period between samplings (in days). Feed conversion ratio (FCR) was calculated by related feed consumption to gain in weight of fish.

$$Feed\ conversion\ ratio = \frac{Feed\ intake\ (g)}{\text{Weight gain (g)}}$$

The mean body weight (g) was calculated by the following formula

$$\text{Mean body weight} = \frac{\text{Total wet weight of the fish in the aquarium } (g)}{\text{Number of fish in the aquarium (g)}}$$

Challenge Test

After 60 days of feeding, 6 fishes were collected from each treatment were challenged I/P with 0.1 ml fresh culture suspension containing $3x10^9$ cells ml^{-1} of *V.parahaemolyticus*. The challenged fish were kept under observation for 15 days and the mortality was recorded.

Results and Discussion

Screening and Identification of Bacteriocinogenic Strain

From raw milk 4 bacteriocinogenic strains were screened using *Vibrio vulnificus* (MTCC 1145) as indicator. The S1 strain showed maximum inhibitory activity. The 924bp 16S rRNA sequence of isolate was submitted to GenBank database with the accession no. KT008282. Similarities between the 16S rRNA sequence of isolate and those of *B. cereus* strains in the GenBank database were 99.0%, which proved the initial identification. The constructed phylogenetic tree using neighbor-joining method further demonstrated that isolate was closely related to the *B. cereus* strain (GenBank accession no. KF601958.1) (Figure 1).

Crude bacteriocin was produced by salting out and dialysis and it was subjected to SDS PAGE which had a molecular mass of 11kDa.

B. cereus produced bacteriocin can withstand the wide range of pH (3-10). Maximum zone of inhibition of 28 mm for *Vibrio vulnificus* was observed at pH 6. There is significant activity was viewed from pH 3 to 10. Similarly, the bacteriocin showed resistant to broad range of temperatures and relating the maximum activity at 40 ^{0} C (22 mm for *Vibrio vulnificus*). Up to 100 ^{0} C purified bacteriocin was stable for 2 hours with substantial antimicrobial activity.

The hydrobiological parameters like Temperature 28.8 ± 10^0C, pH: 7.8 ± 0.6, Water Hardness: 4.12mg/L and DO: 4.13±0.7ml/l^{-1} was estimated. Specific growth rate, feed conversion ratio and survival rate of the *T.trichopterus* fed with four diets were presented in Table 2. The highest weight gain (4.14±0.80) was observed in experiment (10% *B.cereus* diet) which was statistically significantly (p□0.05) higher than control (without Bacteriocin) 3.25± 0.72.

Specific growth rate was increased with the addition of Bacteriocin in fish diets. The fish fed diets contain 10% of *B.cereus* bacteriocin diet has significantly (p□0.05) enhanced feeding and growth rate as compared to fish fed the control diet. In the present study the FCR was higher in control (1.15±0.02) and less in test (0.58±0.13). The survival rate of juvenile *T. trichopterus* was 50% for control and 94% for test.

Challenge Test

The survival percentage was 83.33% of *T.trichopterus* fed on the diet containing *B.cereus* after 60 days and challenged by pathogenic *V. parahaemolyticus* (0.1 ml of $3x10^9$ cells ml^{-1}). But the control diet fed fishes showed less (33.33%) survival than the experimental diet fed *T.trichopterus.*

The priority for the search of alternative protein sources to fish meal in aquaculture research is due to the alarm regarding the future availability of fish meal for incorporation into fish diets (Mondal et al 2008; Hardy 1996). The present study is the evidence for that dietary supplementation with *B.cereus* influenced the feeding and growth parameters in *T.trichopterus*. These results may possibly due to the improved feed intake and nutrient digestibility. Moreover, the bacteriocin of *B.cereus* promotes fish growth.

Overlapping characters in some bacterial species do not consent to its characterization that generally stated in the paradigm of phenotypic identification. But the advancement in molecular biology made the researchers to study, identify, and characterize organisms by using refined markers like rRNA. Ribosomal gene markers predominantly 16S rRNA, are extensively used in molecular analysis to spot and renovate the evolutionary history of microorganisms (Lane et al. 1985; Witt et al. 1989; Nakatsu et al. 2000; Saldarriaga et al. 2004; Steindler et al. 2005).

In contrast with phenotypic identification, even for rare isolates the 16S rRNA sequencing provides explicit data that are reproducible in any laboratories (Hossain 2008). The results of the present study evidently specify the utility of the 16S rRNA sequence analysis in the generic level identification of rare isolate.

Todorov and Dicks (2005) purified the crude protein by precipitating with ammonium sulphate and dialysed against phosphate buffer (pH 6). By gel filtration chromatography it was further purified and then lyophilized. The use of cation exchange resin is appropriate for their purification because of the bacteriocins have positive charge at neutral pH.

The bacteriocin with the low molecular weight was produced by *B. cereus*. This result is accord with the SDS- PAGE of bacteriocins produced by *B. cereus* (Rowaida et al 2009), *B. megateirum* (Svetoch et al 2006) where the estimated molecular weight of bacteriocin is in the range from 4-6 KDa and 3.496 to 6.512 KDa. Teixeria et al (2009) also stated that bacteriocin of *B. circulans* had an approximate molecular weight of 3.5KDa.

The antimicrobial activity utmost was observed at pH 6 but the bacteriocin of *B.cereus* could endure the pH of 3 to 10. Bromberg et al. (2004) stated that the antimicrobial activity of the bacteriocin can be hold in part at the time of acidic to basic shift.

Bacteriocin stability at different pH scale is a restrictive one to use in food items. The antimicrobial activity of bacteriocins produced by *L. plantarum* and *L. brevis* OGI holds its activity in an acidic pH range of 2.0

to 6.0 whilst inactivation occurred at pH 8.0 to 12.0. Crude bacteriocin of *L. lactis* production could be maximized by maintaining the pH of growth media at 6.5. The outcome of pH on bacteriocin production has been well predicted where growth at controlled pH results in higher bacteriocin titers (Yang and Ray, 1994; Franz et al. 1997).

The purified bacteriocin was stable up to 100^0C for 2 h with considerable antimicrobial activity. Thermo stability of bacteriocin at high temperature makes it possible to sterilize the food products even at room temperature, thus avoiding their storage at low temperature. Earlier studies revealed that bacteriocins produced by *L. paracasei*, *L. lactis*, *L. plantarum* and *L. pentosus* remained active after heating till 121°C for 20 min (Papanthanasopoulos et al 1997).

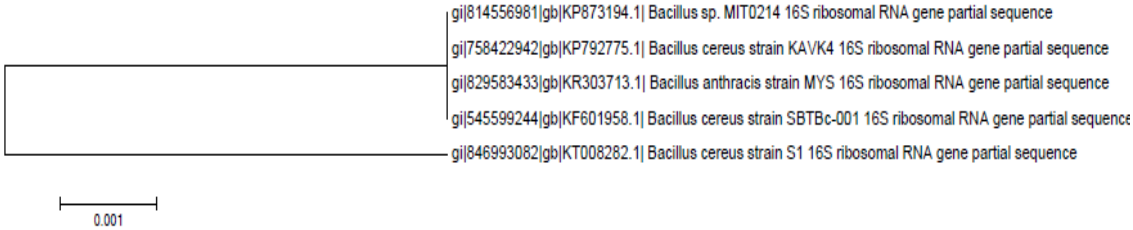
Table.1 Formulation and Proximate Composition (g/100g) of Ingredients in Formulated Feed

S.No	Feed ingredients (g)	Diets in %	
		Control	Test
1.	Fish meal	47.10	46.10
2.	Groundnut oil cake	25.62	22.21
3.	Soya meal	10.34	8.62
4.	Rice bran	8.21	7.82
5.	Tapioca powder	3.24	2.81
6.	Wheat flour	4.89	4.85
7.	Vitamins and minerals	0.50	0.52
8.	Vegetable oil	0.10	0.10
9.	Bacteriocin	0.00	10.00

Table.2 Growth Performance and Survival (%) of Blue Gourami Fish (*Trichogaster trichopterus*) Fed in Experimental Diets

Experimental diets	Initial weight (g)	Final weight (g)	Weight gain (g)	Specific growth rate (%)	Feed conversion ratio (FCR)	Survival rate (%)
Control	2.21±0.12	4.82±0.12	2.60±0.14	0.67±0.21	1.06±0.02	50
Test	2.23±0.14	5.83±0.21	3.14±0.10	0.75±0.24	0.61±0.13	94

Figure.1 Phylogenetic Tree of 16s rRNA for Strain *B.cereus* and the Closest Strain of *Bacillus* using Neighbour Joining Method



Irianto and Austin (2002a) stated that probiotics are the microorganism or its products which afford health benefit to the host and it is used in aquaculture as a growth enhancer and disease controller by enhancing or even replacing the use antimicrobial compounds. The mode of action of the probiotics is rarely investigated, but possibilities comprise competitive exclusion, i.e. the probiotics inhibit potential pathogens colonization in the digestive tract by antibiosis or by competition for nutrients and / or space, alteration of microbial metabolism, and/or by the stimulation of host immunity. Probiotics excite appetite and perk up nutrition by vitamin production, detoxification of compounds in the diet, and by the breakdown of indigestible components. Consequently the use of probiotics can enhance the nutrition level of aquacultural animals and improve immunity of cultured animals to pathogenic microorganisms. In addition, antibiotics usage can be reduced and recurrent outburst of diseases can be prevented.

In the present study, *B.cereus* showed inhibitory effects *in vitro* and against *Vibrio vulnificus*. *B.cereus* fed fish revealed the survival rate of juvenile *T. trichopterus* was 94% of feed among *T.trichopterus* challenged by *V.vulnificus* compared to survival rate 50% in fish fed on diet not supplemented with *B.cereus*.

Specific growth rate was increased with the addition of Bacteriocin in fish diets. The fish fed diets contain 10% of *B.cereus* bacteriocin diet has significantly ($p \square 0.05$) enhanced feeding and growth rate as compared to fish fed the control diet. In the present study the FCR was higher in control (1.15±0.02) and less in test (0.58±0.13). Different researchers like this, have done and similar result were expressed that adding probiotics to diet of different kinds of fish, increase their growth. In the study by Giri et al. (2013) *Lactobacillus plantarum* in three concentrations was added to *Labeo rohita* diet, daily weight growth (WG) and feed conversion ratio (FCR) showed a significant increase and SGR in 10^8, 10^{10} CFU g^{-1} had a significant increase. Similar studies also by Sun et al. (2011) on *Epinephelus coioides*, Aly et al. (2008) on *Tilapia nilotica* (*Oreochromis niloticus*), Suzer et al. (2011) on gilthead sea bream (*Sparus aurata*), Al-Dohail et al. (2009) on *Clarius gariepinus*, Venkat et al. (2004) on *Macrobrachium rosenbergii* obtained similar results.

In conclusion the study reveals that the growth of *T. trichopterus* can be improved and mortality rate was decreased by supplementing the fish feed by *B. cereus*.

In conclusion the study reveals the *B.cereus* can help to improve disease resistance in fish. However their effectiveness may vary among facilities and production units because of the influence of many confounding factors. The probiotic property of isolated *B.cereus* could survive in low pH and can withstand broad range of pH thus indicating that they could be considered as novel probiotic candidates for use in the ornamental fish culture.

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