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Influence of probiotic bacterium *Bacillus cereus* isolated from the gut of wild shrimp *Penaeus monodon* in turn as a potent growth promoter and immune enhancer in *P. monodon*

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ABSTRACT

A probiotic bacterium isolated from the gut of wild shrimp *Penaeus monodon* rendered maximum antagonistic activity against shrimp pathogens and was capable of producing extracellular enzymes. The probiotic bacterium was identified as *Bacillus cereus* through 16S rRNA sequencing. The lyophilized *B. cereus* was supplemented with shrimp basal diet at four different concentrations (0.1–0.4%/100 g feed) in D1–D4 diets. The viability of probiotic bacterium in the test diets was evaluated during the study period at various time intervals. The viability ranged from 50.24 ± 1.42 to 180.34 ± 1.30 CFU/g in D1 to D3 diets on the 30th day, whereas it was slightly declined from 45.23 ± 1.30 to 169.13 ± 1.18 CFU/g during the 90th day of storage. A control diet (C), devoid of probiotic supplementation was also simultaneously prepared. During experimentation, *P. monodon* postlarvae (PL-15) were cultured in individual one tonne capacity FRP tanks in triplicates provided with equal amount of substratum (clay soil) and fed with these respective diets at *ad libitum* for 90 days. Survival was high (82.0 ± 1.60%) in D4 diet fed shrimp as against a low survival of 65.0 ± 1.33% displayed by control diet fed shrimp. Overall growth responses inferred that a maximum production of 10.45 ± 0.275 g, SGR of 4.40 ± 0.179% and a better FCR of 1.27 ± 0.081 were obtained in D4 diet fed shrimp. However, the water quality parameters showed nonsignificant ($P > 0.05$) variations among the control and the probiotic treated groups. The tested immunological parameters such as Total haemocyte count, phenoloxidase activity, respiratory burst activity, lysozyme activity, plasma protein concentration and bactericidal activity were higher in D4 diet fed *P. monodon*, when compared to that of other diets fed shrimp. It is therefore suggested that lyophilized probiotic *B. cereus* at a concentration of 0.4%/100 g feed was efficient in stimulating the growth and immunity in shrimp.

1. Introduction

Probiotics application in aquaculture sector is gaining importance, with the development of eco-friendly aquacultural practices [1]. Probiotic may be defined as a live microorganism that offers a beneficial effect in the host when ingested in required amounts [2]. In aquaculture, administration of probiotics can be done through dietary supplement or as a water additive [3]. Antibiotic treatment for disease control measure during aquacultural practices has several negative effects such as development of drug resistant bacteria and low efficiency of antibiotic treatment for diseases [4]. Moreover, they cause a deleterious effect on the environment [5,6]. Hence an effective alternative for the usage of antibiotics could be the application of probiotic which would favour the environment [7,8].

Probiotics have a wide range of beneficial effects that include water quality improvement, enhancement of immune responses in host species; competitive exclusion of bacterial pathogens through the production of inhibitory compounds; enhancing the nutritional status of the host species by producing supplemental digestive enzymes etc. [8,9]. In fish and shellfish culture, photosynthetic bacteria, yeast, *Bacillus* sp. and *Lactobacillus* sp. have been evaluated

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[1,10,11]. *Bacillus* had been extensively used as efficient probiotics [12] due to the capability that they secrete a wide range of exo-enzymes and antimicrobial compounds [3,13]. Probiotics administered as live supplement in diets must be capable of surviving and pass through the intestinal tract. Probiotic application in the diet break down the toxic and non-nutritious components of the diet and facilitate the digestion of the host by preventing the colonization of pathogens in the gut by providing antimicrobial compounds, excluding them for nutrients and mucosal space [14]. Considering the paucity of informations on the efficacy of host species dependent gastrointestinal probiotic microbes in terms of growth promotion and immune modulation in widely cultured Indian tiger shrimp *Penaeus monodon*, the present investigation was carried out.

2. Materials and methods

2.1. Isolation, enzyme production and identification of probiotic bacterium

The probiotic bacterium used in the study was isolated from the gut of wild shrimp *P. monodon* obtained from Manakudy estuary, Tamilnadu, India. Altogether eight morphologically different bacterial colonies were obtained and all these bacterial isolates were screened for enzyme production on respective agar plates i.e. protease, amylase, lipase and phytase activity of the bacterial colonies were determined using skim milk agar, starch agar, sprit blue agar and phytase specific medium, respectively [15]. The antagonistic activity against shrimp pathogens was determined by agar well diffusion method [16]. Based on the performance of best production on the above enzymes and antagonistic activity, only one organism was chosen. Initially this potent organism was identified up to genus level through morphological and biochemical characteristics using Bergy's manual of systematic Bacteriology [17]. Further, this probiotic bacterium was molecularly identified by 16S rRNA sequencing. Then it was mass cultured for 48 h in MRS broth using an orbital shaker at 150 rpm. After incubation, the cells were harvested by centrifugation and then they were subjected to lyophilization using a lyophilizer (VirTis, -25- SRC- 3SP) and stored in a deep freezer until further use.

2.2. Antimicrobial activity

The antimicrobial activity of *Bacillus cereus* against shrimp pathogens was performed by agar well diffusion method. The shrimp pathogens such as *Vibrio harveyi*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* and *Aeromonas hydrophila* were collected from Microbial culture collection centre, Centre for Marine Science and Technology, M. S. University, India. The shrimp pathogens were isolated from diseased shrimps and was identified upto species level using Probabilistic Identification of Bacteria (PIB) software package [18] and an implementation of Bayers' theorem by Willcox et al. [19,20]. Before the start up of experiment, selected shrimp pathogens were cultured in Zobell marine broth overnight at 32 °C. *B. cereus* was cultured in MRS broth for 48 h at 32 °C. After incubation period, 2 ml of broth culture was taken and centrifuged at 10,000 rpm for 15 min. The cell free supernatant (50 µl) was taken and loaded into the well of Muller Hinton agar plates, previously coated with overnight culture of individual shrimp pathogens. After 24 h of incubation, the zone of inhibition was measured and expressed in AU/ml.

$$\text{Arbitrary Unit(AU/ml)} = \frac{\text{Zone of inhibition(mm)}}{\text{Volume of the sample loaded}} \times 1000$$

2.3. Diet preparation and experimentation

Four experimental diets were prepared by supplementing the lyophilized probiotic strain at 0.1, 0.2, 0.3 and 0.4% concentrations along with the basal diet and the diets were designated as D1, D2, D3 and D4, respectively. A control diet (C), which devoid of lyophilized probiotic strain was also simultaneously prepared. The basal diet was formulated using fish meal, soybean meal, wheat bran, ground nut oil cake, and tapioca powder with appropriate quantities (Table 1). The ingredients were mixed well with sufficient quantity of distilled water and made as dough. Then the dough was steamed for a duration of 15 min in a pressure cooker, after cooling the dough, the respective concentrations of lyophilized probiotic strain was added along the basal feed. The additives such as vitamin and mineral, cod liver oil and gelatin were also added in the diets, mixed well and pelletized using a hand pellitizer. The prepared diets were then dried in a hot air oven at 45 °C for a duration of 48 h and then stored in individual air tight plastic containers.

2.3.1. Evaluation of viability of probiotic strain in experimental diets

The viability of *B. cereus* in test diets (D1–D4) was evaluated during the storage intervals of 30th, 60th and 90th days at room temperature. Each 10 mg of feed pellets of experimental diets were taken and ground well individually using 1 ml phosphate buffered saline (pH – 7.2) using a mortar and pestle under sterile condition. Then they were serially diluted and spread plated on MRS agar plates and placed in an incubator for 48 h. The bacterial colonies were counted for testing the viability. The assay was carried out in triplicates.

2.3.2. Larval collection and maintenance

Healthy *P. monodon* postlarvae (PL-15) were procured from Aquamarine hatchery, Pondicherry, Tamilnadu, India and transported to the laboratory carefully in oxygenated polythene bags. The postlarvae were acclimatized in 5 tonne capacity FRP tank with 25 ppt filtered seawater for a period of 10 days. The PL were fed twice a day at *ad libitum* on starter feed (CP Nova). The unfed remains and faecal matters were siphoned out every day prior to water exchange.

2.4. Culture system

The culture experiment was carried out in an out door culture system. The out door culture system consisted of fifteen tanks, out of which three tanks served as the control (C) and the rest of the twelve tanks served as the experimental i.e. (D1–D4) and in total

Table 1
Feed ingredients used for the preparation of control (C) and experimental (D1–D4) diets.

Feed ingredients	Type of feed/amount of feed ingredients				
	Control	D1	D2	D3	D4
Fish meal (g)	46	46	46	46	46
Ground nut oil cake (g)	25	25	25	25	25
Soya meal (g)	13	13	13	13	13
Wheat bran (g)	7	7	7	7	7
Seaweed powder (g)	2	2	2	2	2
Tapioca powder (g)	3	3	3	3	3
[b]Vitamin and mineral (g)	2	2	2	2	2
Cod liver oil (g)	2	2	2	2	2
[a]Gelatin (g)	2	2	2	2	2
Lyophilized probiotic strain (g/100 g)	–	0.1	0.2	0.3	0.4

[a] Binder.
[b] Supradyn (multivitamin tablet with minerals and trace elements).