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ARTICLE

Effect of a three-component bacterial consortium in white shrimp farming for growth, survival and water quality management

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ABSTRACT The effect of a bacterial consortium containing *Rhodopseudomonas palustris* SUP-2, *Bacillus subtilis* SUP-3, and *Bacillus firmus* SUP-1 strains for white shrimp (*Penaeus vannamei*) farming were investigated. Shrimp growth, water quality, survival rate and enzymatic activities were followed at different stages of farming. Consortium developed using equal proportion of each bacterium was added to shrimp ponds at different concentrations. Dosages were adjusted in three different stages, first 40 days (10 l/ha), second 40 days (15 l/ha) and then continued up to harvest (20 l/ha). Consortium was used in the experiment ponds and no consortium was used in the control ponds. The mean survival rates were significantly lower, 65.1 ± 1.4% and 67.8 ± 2.2% in control ponds BPC-1 and BPC-2 ($P<0.001$), respectively. The statistical analysis showed significant differences ($P<0.05$) in the weight of the animals between the treatment (34.98 ± 0.1 in BTP 1 and 35.26 ± 0.1 g in BTP 2) and control (29.23 ± 0.1 in BCP 1 and 30.41 ± 0.1 in BCP 2) groups.

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KEY WORDS

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Introduction

Aquaculture and particularly shrimp culture has become a pivotal economic activity in many countries. An intensive culture system is commonly used for shrimp cultivation, because it produces higher yields than other systems (Shang et al. 1998). Since intensive shrimp culture needs high volume of pelleted feed and other additives, inadequate management may cause degradation of pond water environmental parameters including accumulation of black and glutinous sediment, called as organic sludge, in pond bottom soil (Shang et al. 1998; Rosenberry 1993). The bio-remediating agents used in shrimp aquaculture are predominantly photosynthetic bacteria (PSB) having antagonistic activity against shrimp pathogen at the same time improving water quality (Vine et al. 2006; Kesarcodi-Watson et al. 2008). Recently, an approach of using combined probiotics (microbial consortium) is gaining popularity worldwide (Wang and Wang 2008). It is proposed that application of beneficial microorganisms to the pond soil can accelerate decomposition of undesirable organic matters and other waste products through bioremediation and perhaps even increase ambient levels of oxygen (Wang and Han 2007; Sonnenholzner and Boyd 2000a).

The antibiotic resistance and its epidemiological magnitudes led to the investigation of several alternate approaches for disease management in aquaculture systems. Amongst them the most scientific, eco-friendly and cost-effective approach is the use of probiotics as prophylactics. The microbiota in the gastrointestinal tract (GIT) of shrimps can be modified by ingestion of other microorganisms; therefore, microbial manipulation establishes a practical tool to reduce or eliminate the prevalence of opportunistic pathogens (Balcazar et al. 2006). Natural production of some substances (Dinh et al. 2010; Iyapparaj et al. 2013) by bacteria in flocculating condition has been reported to prevent growth of co-habiting pathogenic species such as *Vibrio harveyi* (Defoirdt et al. 2007; Halet et al. 2007). Species in the genus *Vibrio* constitute the major bacterial pathogens in cultivated shrimp worldwide (Lightner 1993), and also *Aeromonas* that have been considered as foremost pathogens in aquaculture systems (Sindermann 1990).

The potential benefits of addition of bacterial consortium

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in shrimp ponds include upgrading of water quality, enhancement of nutrition of host species through the production of auxiliary digestive enzymes, lower incidence of diseases and greater survival, and improved immune protection system (Verschuere et al. 2000). In the year 2003, Gamboa-delgado and co-workers reported that amylase activity increased the shrimp body weight. Maugle and co-workers (1983b) reported incremental increases in growth with increasing amylase supplements.

The aim of this study was to determine the effect of bioremediating isolates derived from brackish water on water quality parameters and the spectrum of antagonism against *Vibrio* and *Aeromonas* shrimp pathogens. This study was also involved digestive enzyme parameter assays and characterization, where protease and amylase were used as indicators.

Materials and Methods

Chemicals, media and reagents

All chemicals, media and reagents used in this study were of analytical grade and procured from Rankem and Hi-Media (India).

Sampling site and sampling procedure

Water samples were collected from the center of the Junput (21.68°N and 87.55°E) at Purba Midnapore district (West Bengal, India). Water depth (measured from the bottom of morphological structures) ranged from approximately 5 to 20 cm, depending on low wave. Water at the collection sites had temperature between 27 to 33 °C, salinity 14 ppt and pH 7.8. Each water sample (100 µl) was spread directly onto agar plates (containing tryptone 1%, yeast extract 0.5%, NaCl 0.5% and agar 1.5%, pH 7) at the study site. Water samples were also carried to the laboratory in sterile plastic containers within an hour at ambient temperature for further analysis. One ml of water sample had been serially diluted in sterile distilled water to get a concentration range between 10^{-1} to 10^{-6} dilution.

The bacterial isolates had been further sub-cultured on the respective media, in order to obtain pure culture and among them three isolates (SUP-1, SUP-2 and SUP-3) were selected based on their prevalence and antagonistic activity against shrimp pathogen.

Identification of the isolates

The preliminary morphological characteristics were determined by bright field microscopy (Zeiss Axiostar Plus, Zeiss, Germany). All isolates were also evaluated by conventional biochemical tests (Bergey's Mannual 2014). Scanning electron microscopy was conducted for selected isolates. The bacterial culture was smeared on cover slip, fixed with *p*-glutaraldehyde (4% v/v) and dehydrated (ethanol gradient: 30, 50, 70, 90 and 100% v/v for 15 min on absolute ethanol). Then it was coated with gold spattering and examined under SEM (Carl Zeiss SMT AG, Germany). Molecular identification of microbial isolates was performed by sequence comparison of amplified 16S rDNA region using primers and conditions as reported earlier (Ray Chaudhuri and Thakur 2006).

Screening of potent amylase producer

To find out potent amylase producing bacterial strains using modified medium containing (g/l) starch 10, peptone 10, yeast extract 20, KH_2PO_4 0.50, CaCl2.2H2O 0.50, agar 10, pH 8 at 32 °C temperature for 24 h. After incubation, 1% iodine solution (freshly prepared) was flooded on the starch agar plate. Presence of blue color around the growth indicates negative result and a clear zone of hydrolysis surrounded the growth, indicates positive result. Based on the highest clear zone to colony size ratio (cz/cs), bacterial colony was selected and designated as SUP-2. The isolate was preserved at 4 °C (in plate, stab and liquid form) and sub cultured at a regular interval for further use.

Media composition and fermentation condition

Amylase production by the isolate (SUP-2) was carried out in Erlenmeyer flasks (250 ml) contained 100 ml fresh liquid medium (modified medium containing (g/l) starch 10, peptone 10, yeast extract 20, KH_2PO_4 0.50, $CaCl_2.2H_2O$ 0.50). The physicochemical and cultural conditions were optimized by studying one variable at a time (OVAT) approach, where one factor is varied keeping others constant. The effect of physical parameters like, *e.g.,* incubation period (6-24 h), temperature (20-60 °C), pH (5.0-9.0) and chemical parameters like carbon sources (glucose, galactose, lactose, maltose, mannose, starch, sucrose) nitrogen sources (yeast extract, urea, peptone, beef extract, NH_4Cl, NH_4NO_3, KNO_3 and $NaNO_3$) and phosphate sources (KH_2PO_4, K_2HPO_4, Na_2HPO_4, NaH_2PO_4 and $(NH_4)H_2PO_4$) were evaluated for amylase production. The whole optimization process was carried out using freshly grown SUP-2 strain (A620~1.63). The fermented medium was centrifuged at 5000 rpm for 5 min and the amylase activity in the supernatant was measured through dinitrosalicylic acid method according to the protocol of Maity et al. (2011).

Antimicrobial sensitivity test

Different pathogenic strains from Tryptone Soy Agar (TSA) slants were inoculated into Tryptone Soy Broth (TSB) and incubated at 30 ºC for 24 h to prepare young cultures. The cul-

tures of pathogenic strains, *Vibrio parahaemolyticus* (MTCC 451), *Vibrio harveyi* (MTCC 1639) and *Aeromonas veronii* (MTCC 3249) were diluted 10^{-3} times using sterile Normal Saline Solution (0.9% NaCl) to reach the concentration of 10^{-6} cfu ml^{-1}. Antimicrobial sensitivity test was performed using agar-well diffusion method. After preparation of Tryptone Soy Agar plate the test organisms were swabbed on the Petri plates and left for 10 min. Next to that the wells were made on the plate and the crude centrifuged (5000 rpm) fermented medium was aspirated and loaded into the wells with the capacity of 100 µl each. The Petri plates were incubated overnight without inverting at optimum temperature of 32 °C.

Experimental design

The study was conducted from March 21, 2015 to August 13, 2015 at Contai shrimp ponds, located in the East Coast of the Bay of Bengal Sea. Four shrimp ponds were selected with two treatments (BPT-1 and BPT-2) and two controls (BPC-1 and BPC-2). The formulated (ECO-PRO) consortium (SUP-1, SUP-2 and SUP-3 strains in equal concentration) was added into the treatment ponds and not into the control ponds. The maximum depth was 120 to 130 cm with similar morphometric and size features (0.34-0.36 ha). The ponds had been used for four culture cycles and therefore, were considered aged ponds. The management and husbandry process was slightly modified, described by Corre et al. (2000). The pond bottom sun dried, desilted and was sanitized using lime stone power with soil conditioner (WitaMin, Biostadt India, Mumbai, India) prior to stocking. All the ponds were filled with cloth-filtered seawater (80, 100, and 120 meshes) having 15 ppt salinity after 10 days solarization. Each pond was stocked with *Penaeus vannamei* PL 12 (Vaishaki Biomarine, Vishakapatnam, India) at a density of 50 pcs/m^2. Shrimps were fed with commercial pellets (CP, India) twice a day for the first 30 days at a rate of 5-10% of the shrimp body weight and after that Automatic Feeder (Aron, Thailand) was used up to harvest. Paddle wheel aerators were used 3-4 h for 1st 30 days, 6-8 h for 30-90 days and 10-12 h up to harvest and adjusted the numbers/HP with 400 kg biomass after 30 days.

The probiotics was formulated in liquid form packed in airtight HDPE bottles. The product had bacterial cell densities of $1x10^9$ CFU (colony-forming units) ml^{-1} and contained consortium with equal mixture of SUP-1, SUP-2 and SUP-3. The rate and frequency of application of the formulated consortium probiotics in shrimp treatment ponds was 10 l/ha for first 40 days, 15 l/ha for second 40 days and then 20 l/ha continue up to harvest. Dosage was given in every 10 days and adjusted as per the above schedule.

Water quality sampling

Water pH, temperature and salinity were monitored daily at 06:00-07:00 h and 17:00-18:00 h using KIT (Allvet-Thailand), digital water thermometer (Hicks-Japan) and refractometer (Atago-India), respectively. Other water parameters were analyzed weekly. Unionized ammonia and particulate organic matter (POM) were analyzed following the procedures prescribed by Strickland and Parsons (1972). Cu, B, Zn and Mn were analyzed through Atomic Absorption Spectroscopy (Shimazdu AA7000, Japan). Quantitative analysis of phytoplankton was done using a hemocytometer and a compound microscope following the procedure of Martinez et al. (1975). Presumptive *Vibrio* counts were determined by a spread plate technique on thiosulfate-citrate-bile salt sucrose (TCBS) agar by employing the method of Reilly (1982). The agar plates were incubated for 18-24 h in ambient temperature. Yellow and green colonies were counted after 24 h and were assumed to be *Vibrio*. Luminescent colonies were counted on TCBS plates in a darkened room.

Table 1. Characterization of three different bacterial isolates isolated from sample site (Junput).

Strains	SUP-1	SUP-2	SUP-3
Physiological characterization			
Temperature tolerance range	10-65 °C	15-60 °C	15-55 °C
Optimum temperature	30 °C	30 °C	32 °C
pH tolerance range	3-11	4-11	3-12
Optimum pH	8.0	8.5	8.0
Biochemical characterization			
Amylase	Positive	Positive	Positive
Catalase	Positive	Positive	Positive
Protease	Positive	Negative	Positive
Urease	Negative	Positive	Positive
Methyl red	Negative	Positive	Negative
Voges-Proskauer test	Negative	Negative	Negative
Indole	Negative	Negative	Negative
Citrate	Negative	Positive	Negative
Maximum identity with organism			
	B. firmus	*R. palustris*	*B. subtilis*

Statistical analysis

Statistical differences among treatments were analyzed using the analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) with the SigmaStat software V.10.0 (SPSS, 1999) at 5% level of significance.

Results and Discussion

Bacterial isolation and identification

Based on their abundance and antagonistic activity against

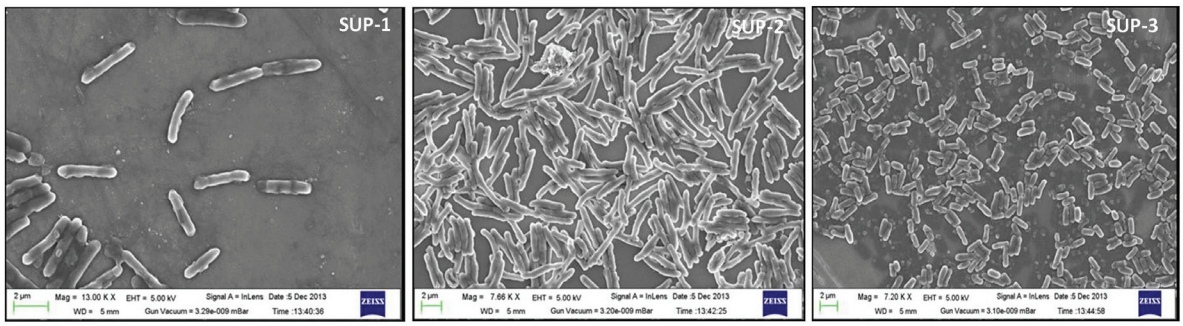

Figure 1. Scanning Electron Microscopic photograph of SUP-1, SUP-2 and SUP-3.

pathogen, molecular characterization was performed, which indicated that SUP-1 and SUP-3 as *Bacillus* sp., where the similarity is greater than 98% (data not shown) and SUP-2 showed 99% similarity with *Rhodopseudomonas* sp. The Gen-Bank Accession Number of the isolates was KT875347, KT875348 and KT875349, respectively. The details of the morphological, biochemical and physiological characterization are presented in Table 1. Scanning electron micrograph of selected strains are depicted in Figure 1 and the neighbor joining phylogenetic tree from analysis of 16S rDNA gene sequence of bacterial isolates is presented in Figure 2.

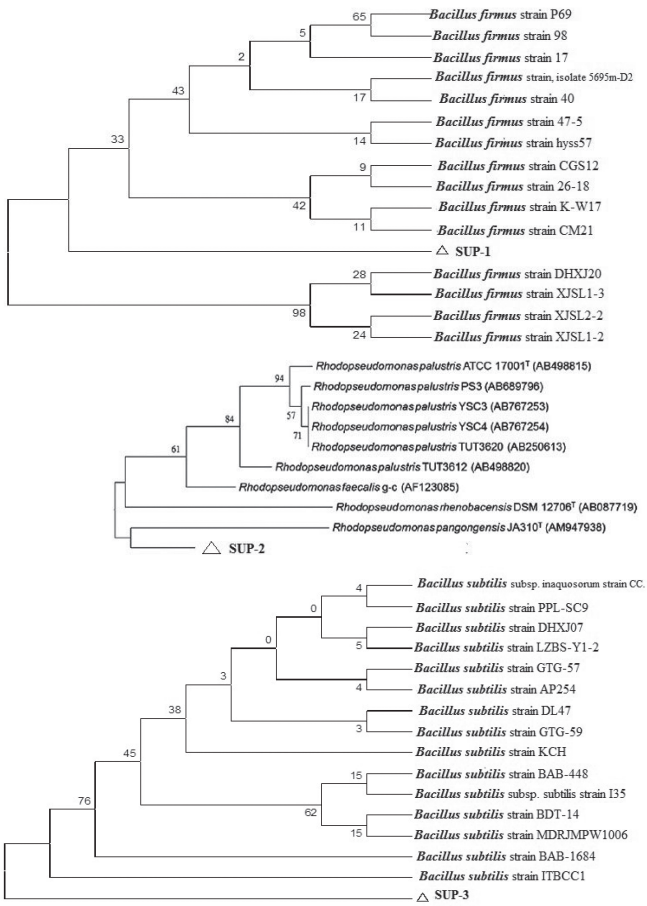

Figure 2. Phylogenetic tree of SUP-1, SUP-2 and SUP-3 strains.

Optimization of α-amylase production

Optimization of α-amylase production in submerged fermentation was performed by using quasi-optimum (OVAT) protocol. Among the different carbon sources used (glucose, galactose, lactose, maltose, mannose, starch, sucrose) α-amylase production was higher with starch as sole carbon source at 1.0% level (Fig. 3a). From the present findings, it is observed that the activation of α-amylase needs substrates having α-1, 4 glycosidic bond, including starch and maltose, but glucose represses its production. The biosynthesis of α-amylase in most species is repressed by readily metabolizable substrates, particularly glucose, by a mechanism of catabolite repression, facilitated by the protein encoded CreA gene (Kato et al. 1996). *Rhodopseudomonas palustris* SUP-2 produced a substantial amount of α-amylase in presence of all the tested nitrogen sources, while highest enzyme biosynthesis of 375.6 U/ml occurred in presence of 0.5% (w/v) beef extract (Fig. 3b). Basic nitrogen is essential component for growth of microbes and production of secondary metabolites. Among these, studied phosphate source (Fig. 3c). $(NH_4)\ H_2PO_4$ with 0.5% (w/v) concentration maximized amylase production (292 U/ml). During OVAT optimization, *R. palustris* SUP-2 was found to produce the highest amount of amylase (363.4 U/ml) after 18 h of fermentation (Fig. 3d). Figure 4e depicted

Table 2. Antimicrobial assay of isolated strains against test organisms (inhibition zone in mm).

Isolated strains	*V. parahaemolyticus*	*V. harveyi*	*Aeromonas veronii*
SUP-1	4.3	1.4	0.4
SUP-2	1.8	2.3	2.1
SUP-3	NIL	0.9	3.2

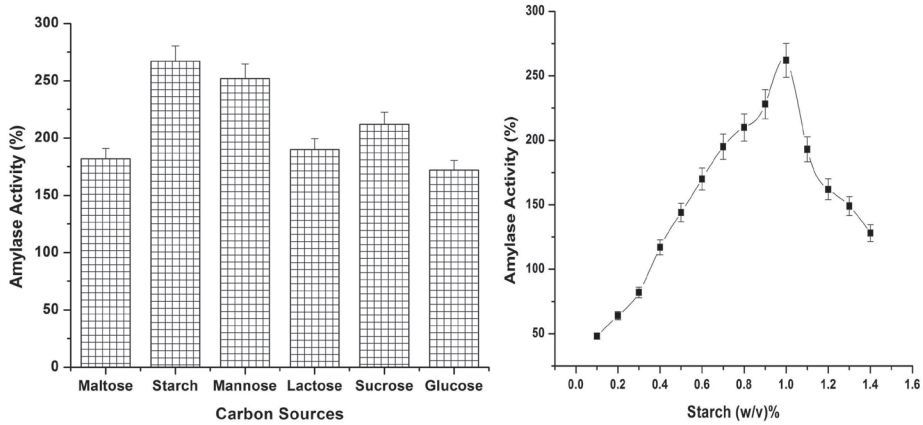

Figure 3a. Effect of different carbon sources and starch concentration (%) on α-amylase production.

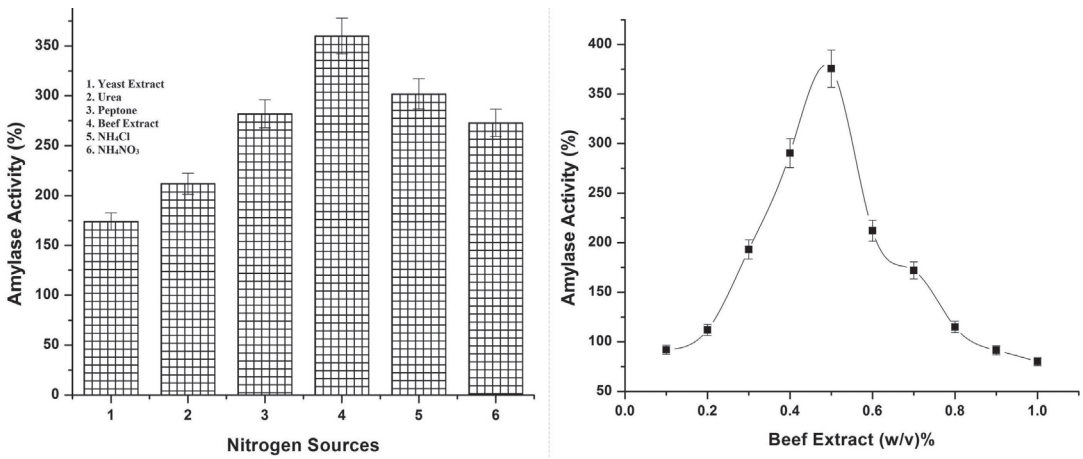

Figure 3b. Effect of different nitrogen sources and beef extract concentration (%) on α-amylase production.

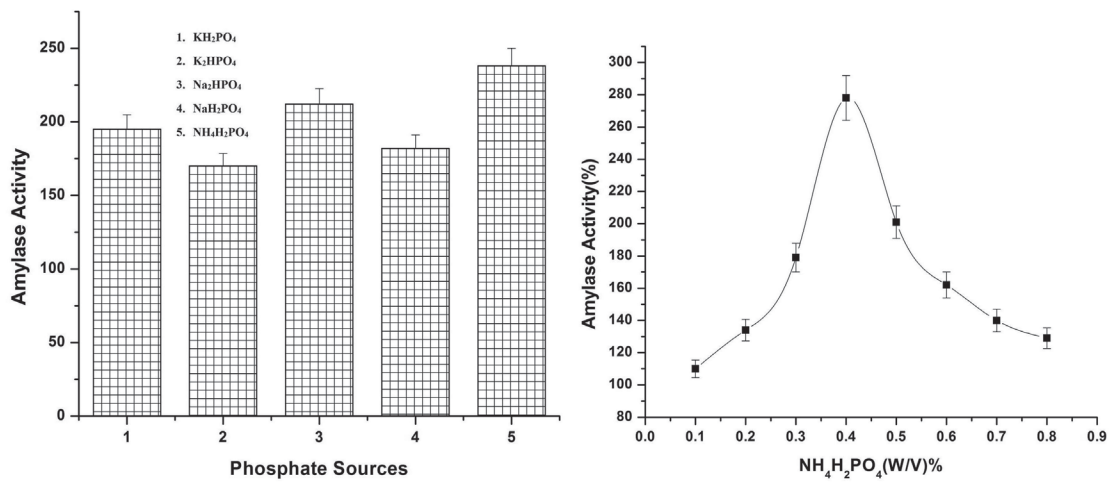

Figure 3c. Effect of different phosphate sources and $(NH_4)H_2PO_4$ concentration (%) on α-amylase production.

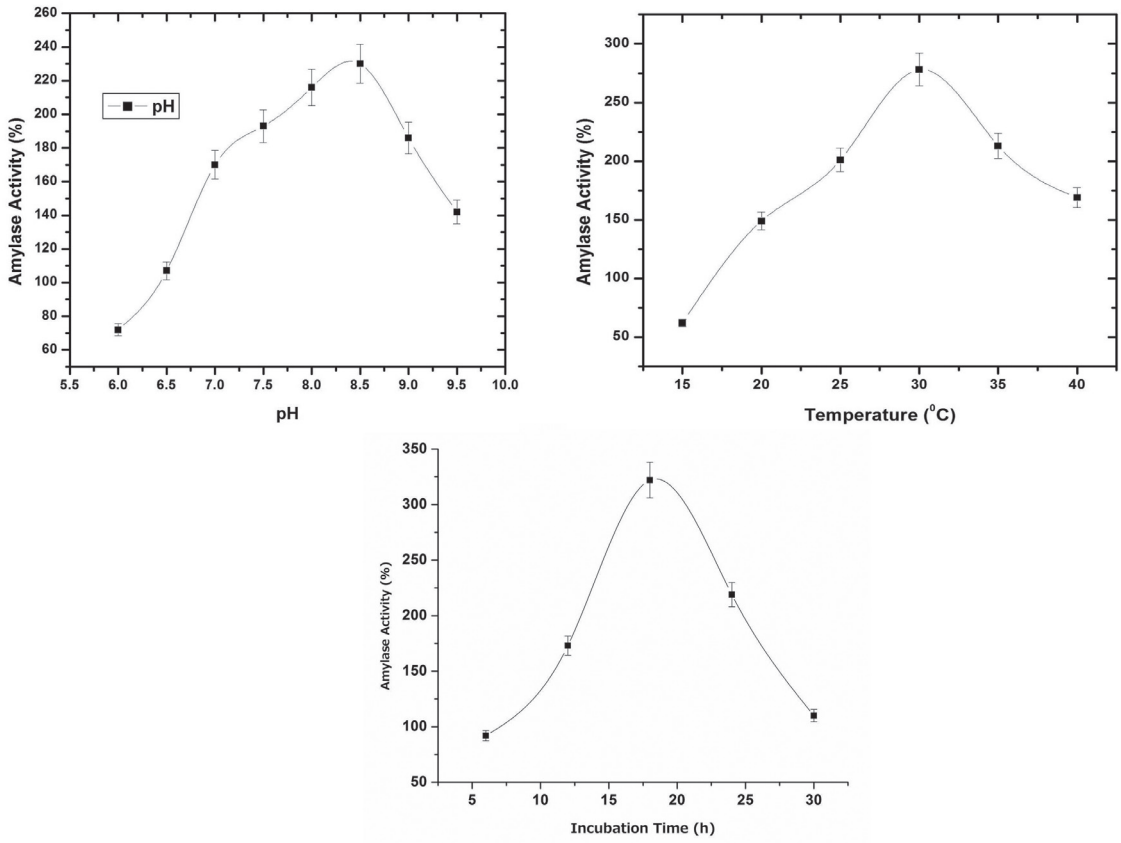

Figure 3d. Effect of pH, temperature (°C) and time (h) on α-amylase production.

the effect of different pH, incubation time and temperature on α-amylase production.

Determination of antimicrobial activity

Antimicrobial activity of the extracellular supernatants against the pathogenic bacteria was determined with the plate diffusion method (Fig. 4) and zone of inhibition represented in Table 2. Antimicrobial activities against test bacteria were observed in plates with pH non-adjusted SUP-1 and SUP-3 cultures. In contrast, slight inhibition was observed in pH-adjusted cultures. In pH-adjusted and pH non-adjusted SUP-2 cultures, strong antimicrobial activities were evident toward all target bacteria. Previous studies reported antimicrobial

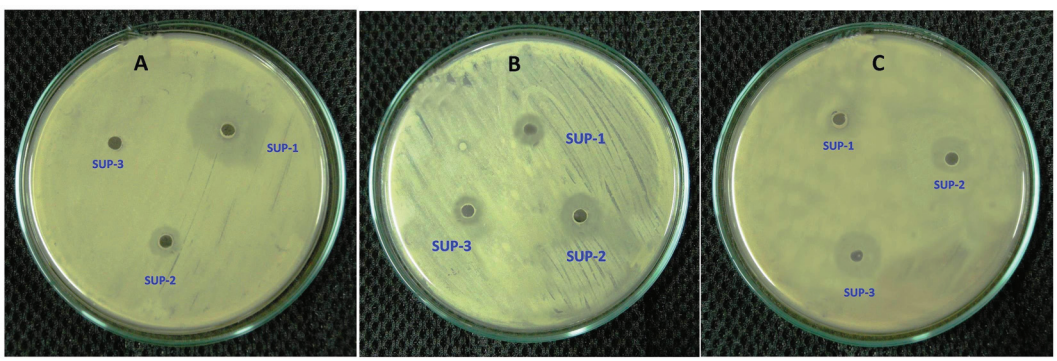

Figure 4. Inhibition by SUP-1, SUP-2 and SUP-3 culture supernatant as measure with the plate diffusion assay. Supernatant were placed on to lawns of (A) *V. parahaemolyticus*, (B) *V. harveyi* and (C) *A. veronii*.

Table 3. Zootechnical results and significant differences in BPC-1 and BPC-2 (control) and BPT-1 and BPT-2 (treatment) ponds.

	BPC-1	BPC-2	BPT-1	BPT-2	Significance
Survival (%)	61.1 ± 1.4	63.8 ± 2.2	69.04 ± 1.4	77.9 ± 2.2	P<0.001
FCR	1.65 ± 0.4	1.69 ± 0.4	1.41 ± 0.4	1.49 ± 0.4	P<0.05
Final body weight (g)	29.23 ± 0.1	30.41 ± 0.1	34.98 ± 0.1	35.26 ± 0.1	Not Significant

effects due to the production of antibiotics, siderophores, bacteriocins, proteases, hydrogen peroxide and lysozyme; changes in pH; and the production of organic acids and ammonia (Nissen-Meyer and Nes 1997; Le Marrec et al. 2000; Verschuere et al. 2000). In spite of boiling the SUP-1 and SUP-3 culture, antimicrobial activity remained same. Reddy (2000) recommended that the pH range of 7.5 to 8.5 was the best for shrimp culture. For this reason the antimicrobial activity of three organisms were tested at pH 8. It was found that SUP-2 showed the antimicrobial activity against all the tested pathogens but SUP-1 and SUP-3 did not showed such antimicrobial activities at pH 8. These results suggested that among the three organisms, SUP-2 was showed the best antimicrobial activity against pathogens. The characterization of inhibitory substance derived from SUP-2 cultures will be resolved in future studies.

Zootechnical results

Table 3. presents the zootechnical results of shrimps reared in both controlled and treated ponds. The mean survival rates were respectively 65.1 ± 1.4% and 67.8 ± 2.2% for BPC-1 and BPC-2 pond (P<0.001). Probiotic treatment was found to significantly improves the survival rate (P<0.001) and the food conversion ratio (P<0.05) (Table 3.). In BPT-1 and BPT-2, survival was higher by 13% and 21% and FCR lower by 15% and 12% compared to control pond BPC-1 and BPC-2, respectively. The treated pond showed that average mean shrimp weights after 117 days were 34.98 ± 0.1 and 35.26 ± 0.1 g and control pond 29.23 ± 0.1 and 30.41 ± 0.1, respectively. The statistical analysis depicted significant differences (P<0.05) in the weight between the treatment and control groups.

Previous studies have suggested that some bacteria may also help in the process of digestion of shrimp by producing extracellular enzymes like, proteases, lipases and carbohydrolases, as well as providing necessary growth factors for controlling the physicochemical parameters of soil and water in the culture ponds (Arellano and Olmos 2002; Ochoa and Olmos 2006; Bandyopadhyay et al. 2015).

Consortium of microorganisms (SUP-1, SUP-2 and SUP-3) induced the best growth performance and antagonistic activity against shrimp pathogens. Similar result was reported by Ghosh et al. (2003) and Wang and Xu (2006).

Environmental parameters of pond

Particulate organic matter (POM) and unionized ammonia were significantly different (P<0.05) in all treatments as compared to control ponds (Fig. 5a-b). The status of unionized ammonia ranged from, 0.001 mg/L to 0.750 mg/L and 0.003 mg/L to 0.160 mg/L, while the POM obtained were 12.56 mg/L and 2.10 mg/L in control and treatment ponds, respectively. POM and unionized ammonia was found to be

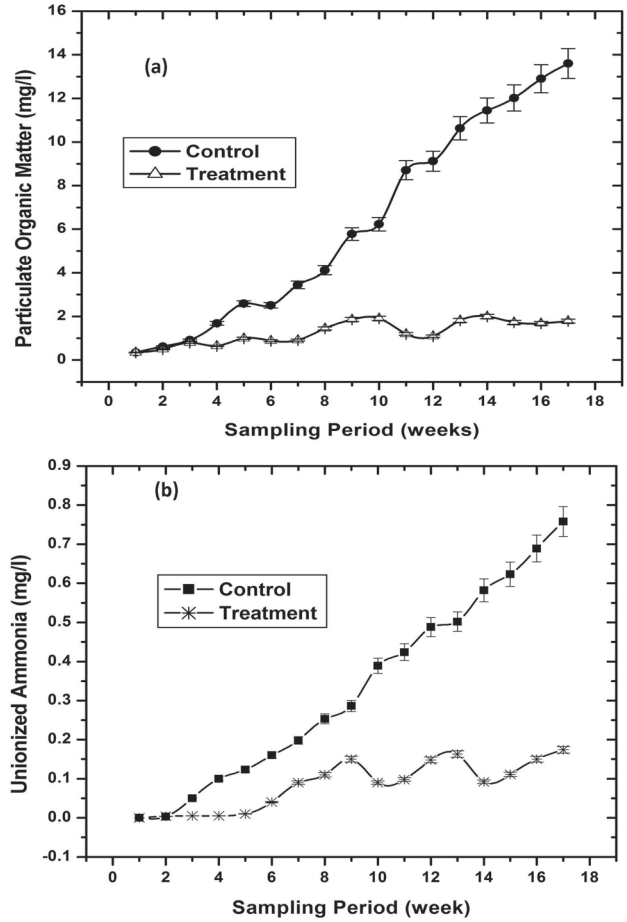

Figure 5. Weekly particulate organic matter level (mg/l) and unionized ammonia level (mg/l) of the pond (control and treated). Each mark represents mean and standard error of three replicates.

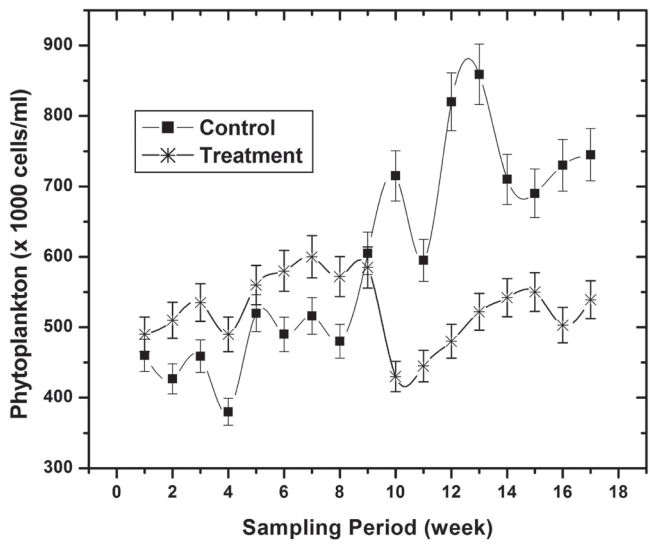

Figure 6. Weekly phytoplankton count (cells/ml) of the control and treated ponds. Each mark represents mean and standard error of three replicates.

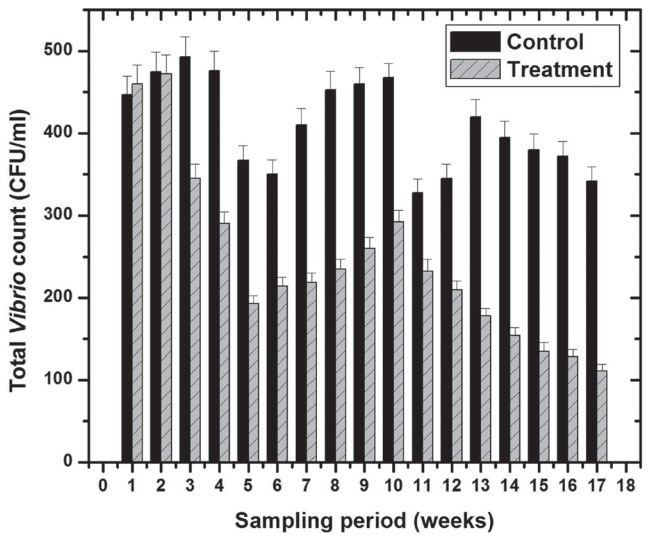

Figure 7. Weekly total reasonable *Vibrio* bacterial counts (cfu/ml) of the pond water. Each mark represents mean and standard error of three replicates.

significantly low and stable in ponds throughout the culture period. Mean phytoplankton count was mostly same among the treated ponds, but significantly higher in the control ponds. However, a relatively stable phytoplankton count was noticed in treated ponds compared to control ponds (Fig. 6). Over population of plankton creates detrimental effect of plankton crash in the pond. It mainly happened due to eutrophication. Simultaneously, *R. palustris* helps sulfur reduction in pond water and stabilize the plankton bloom. The reason behind that bacteria convert ammonium to nitrite and then to nitrate (NH_3, NO_2^-, NO_3^-). The aerobic ammonia-oxidizing bacteria (AOB) oxidize ammonia to nitrite through hydroxylamine and then the nitrite-oxidizing bacteria (NOB) oxidize nitrite to nitrate. Denitrifying bacteria possess several clusters of genes involved in denitrification (Philippot 2002). These genes encoded by four metalloenzymes like nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase, which chronologically reduce nitrate to N_2 (NO_3^-, NO_2^-, NO^-, N_2O, N_2). The phytoplankton density in probiotic treatments was dominated by the beneficial plankton, *i.e.* the diatoms and green algae (*Chlorella*), which comprised about 80-85% of the total plankton. The blue-green algae (*Microcystis*) dominated the phytoplankton in control ponds. Close to end of the culture period, more microcystis and dinoflagellates (*Chattonella*) population was observed. Metals ions were found in negligible amount after end of the culture (data not shown).

The reasonable *Vibrio* counts on TCBS plates were significantly lower ($P<0.05$) in treated ponds (111 ± 10 cfu/mL) when compared with the control ponds (346 ± 15 cfu/mL) (Fig. 7).

The luminous *Vibrio* species present in pond water were ruled out when a specific *Bacillus* species were added (Moriarty 1998). Most bacterial cells are passing in the gut with continuous intrusion of microbes coming from the water and food (Gatesoupe 1999). The routine practice of probiotics enhanced digestion by enhancing the population of beneficial microorganisms, microbial enzyme activity and improved feed consumption (Bomba et al. 2002). Rengpipat et al. (1998) reported that appropriate probiotics applications were shown to better intestinal microbial balance, thus leading to improved food absorption.

Conclusion

White shrimp culture ponds treated with bio remediating consortium had better growth, survival, lower FCR, better soil and water quality parameters than control ponds. It has also been critically observed that bacterial strains SUP-2 (*R. palustris*) had much better effect than other two strains. SUP-2 also showed better inhibitory effect against *V. parahaemolyticus,* which became a big threat for Early Mortality Syndrome (EMS) in white shrimp farming in several South East Asian countries and Mexico. Further research is going on for standardization of dosage and other enzymatic activities for bioremediation.

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