Optimization and Partial Characterization of a Putative Probiotic Bacterium Antagonistic to Vibrios in Shrimp Larval Rearing System

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ABSTRACT: Organic aquaculture utilizing probiotics for disease control and bioremediation has gained global acceptance in recent years. A preliminary study was conducted to isolate indigenous bacteria antagonistic to pathogenic vibrios as putative probiotics for shrimp hatcheries and grow-out systems. These efforts resulted in the isolation of a Gram-positive bacilli after screening thirty one water samples, collected from the coastal areas of the Bay of Bengal. This probiont, identified as *Bacillus licheniformis* exhibited vibriocidal activity *in vitro* against 60% of the *Vibrio* species isolated from the same water samples based on broad spectrum antagonistic and hydrolytic properties. In this study, we looked into the effects of pH, temperature, salinity, surfactants (Triton X114, Tween 80 and SDS) and EDTA on the vibriocidal property of the cell-free supernatant of the probiotic and observed that the antagonistic property was refractory to the surfactants used. The optimum activity of the cell-free supernatant was observed at 30°C, below and above which there was a marked decline in the inhibition. Neutral and alkaline pH ranges favored the antagonistic property while it was negligible at acidic ranges. The metal chelator EDTA nullified the antagonistic property at 0.01 mM concentration, the lowest tested, indicating the presence of a metal active group in the active fraction. The study suggests the potential of the selected bacterial isolate as a putative, antagonistic probiotic useful in the aquaculture systems of the tropics considering its broad antagonistic activity, higher activity in the neutral and alkaline pH and optimum activity at 30°C. Efforts are underway to purify and characterize the antagonistic compound in the cell-free supernatant.

Key words: Probiotic, vibrios, *Bacillus*, shrimp, cell-free supernatant

INTRODUCTION

Vibriosis is probably the major disease afflicting fish, crustaceans, and mollusk aquaculture around the world. A crisis in the shrimp industry over the last few years is due to largely to an increase in virulence of pathogens, especially *Vibrio* spp, together with white spot viruses. Usually, the disease is treated rather than the underlying cause. The interactions of microbes, animals and their environment under the stress of commercial production, and the use of antimicrobial chemicals, especially antibiotics and chlorine, have led to the emergence of more virulent pathogens.[1-3] This not only poses a threat to the food safety in shrimp but also jeopardizes this prospective industry from earning foreign currency for the national economy. In Bangladesh, exports of fresh water shrimp, the second biggest foreign currency earner, to the European Union face a suspension due to the detection of health hazardous antibiotic-nitrofuran since January 2009.[4] The use of beneficial bacteria (probiotics) to displace pathogens by competitive processes and to inhibit their proliferation is a better remedy than administering antibiotics.[5-7]

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The isolation of probiotic bacteria from local environment of Bangladesh was first reported in 2008[8] in a study conducted by these authors. The study discovered some potential probionts from the shrimp hatcheries and grow-out systems of the coastal areas of the Bay of Bengal that showed zones of clearance against pathogenic vibrios, isolated from the same water samples. Two bacteria, identified as *Bacillus* sp. and *Pseudomonas* sp. exhibited vibriocidal activity *in vitro* against 60% and 30% of the *Vibrio* species respectively. The advantage of *Bacillus* as a probiotic candidate is that it constitutes a large part of the microflora of the gills, skin and intestinal tracts of shrimps.[9] Further, Gram positive bacteria like *Bacillus* are unlikely to use genes for antibiotic resistance or virulence from the vibrios or related Gram negative bacteria thereby, makes it an attractive probiotic candidate in the shrimp culture ponds.[10] This study therefore characterizes the maximum vibriocidal potency and growth pattern of the isolated *Bacillus* sp. in different environmental conditions, and thereafter optimizes its spectrum of antagonism as its probiotic potential.

MATERIALS AND METHODS

Identification of the probiotic bacteria: The isolated *Bacillus* sp. was identified at its species level based on the microscopic, cultural and biochemical characteristics.[11]

Optimal temperature, pH and salinity for the antagonistic action of the probiotic bacteria: The optimal conditions for growth and production of antagonistic principle by *Bacillus* was assessed by growing the culture in LB broth at various temperatures (25°C, 30°C, 35°C, 40°C and 45°C), pH (6.0, 7.0, 7.5, 8.0 and 9.0) and salinities (0.5% and 1%) in 100 ml culture volumes at 150 rpm for the period of 7 days in duplicates. Samples for cell count and antagonistic activity were drawn in 1st, 3rd, 5th and 7th day. One milliliter of the culture was processed for evaluating the antagonistic potential against a virulent strain of *Vibrio* sp., previously isolated from the shrimp rearing pond [(12)] by disc-diffusion method.[13] The cell counts were determined by taking the absorbance at 600 nm.

Time course for the optimal antagonistic activity production: For the determination of time course for the optimal antagonistic activity of *Bacillus*, it was inoculated in 20 ml LB broth, pH and salinity of the medium adjusted at the respective optimal values and then incubated on a shaking incubator for 150 rotations per minute (rpm) at the optimized temperature. Samples for antagonistic activity were drawn on 1st, 3rd, 5th, 7th, 9th and 10th day and were processed for evaluating the antagonistic potential by the disc diffusion method as mentioned earlier.[13]

Preliminary characterization of the antibacterial component in cell-free supernatant of the probiotic bacteria: The cell-free culture supernatant of *Bacillus* sp. containing the antibacterial principle was obtained from the culture grown under optimal conditions as mentioned earlier. It was treated with Triton X114 (10%, 20% & 30%), Tween 80 (10%, 20% & 30%), SDS (0.25 mM, 0.1 mm, 0.05 mM and 0.01 mM), EDTA (0.25 mM, 0.1 mm, 0.05 mM & 0.01 mM). After an overnight incubation, antibacterial activity against pathogenic *Vibrio* sp. along with appropriate controls was tested by the disc diffusion method.

RESULTS AND DISCUSSION

Identification of the selected probiotic bacteria: The isolated probiont, *Bacillus* sp. was presumptively identified as *Bacillus licheniformis* following their analysis by biochemical tests (Table 1) after comparing with the standards, as outlined in Bergey's Manual of Systematic Bacteriology.[11]

Optimum temperature for growth and antagonistic action of the probiont: *Bacillus licheniformis* is a mesophilic organism and is able to grow in a wide range of temperatures. Here, we observed that the antibacterial activity of the bacterium appears to be directly proportional to its growth. Its greatest vibriocidal activity and the highest growth both were recorded at 30°C with a

maximum inhibitory zone of 16 mm, observed on the 3rd day of post-harvest (Figure 1A and B). At day 7 of bacterial growth, the activity decreased to 12 mm zone of clearance, an activity still considered to be significant to be a potential probiont.

Table 1. Biochemical characteristics of the probiont

Biochemical test	Reaction
MR	+
VP	+
Indole	-
Citrate	+
Propionate	+
Gelatin liquefaction	+
Nitrate reduction	+
Oxidase	-
Catalase	+
Glucose fermentation	AG
Mannitol fermentation	+
Arabinose fermentation	+
Xylose fermentation	+
Lactose fermentation	+
Starch hydrolysis	+

AG: acid and gas from glucose fermentation; + denotes ability to ferment sugar.

Optimum pH for growth and antagonistic action of the probiont: The tested pH ranges did not result in significant variation in the growth of *B. licheniformis* or inhibitory activity except at pH 6. The antagonistic activity was highest at pH 7.5 after 3 days of cultivation with a clearing zone of 13 mm (Figure 2).

Optimum salinity for growth and antagonistic action of *Bacillus*: The tested salinities of 0.5 and 1.0% did not result in significant variation in the growth of *Bacillus licheniformis*. It showed optimum growth at 0.5% salinity, wherein, peak growth was attained after the 3rd day with concomitant maximum production of inhibitory metabolite. Antagonistic activity was observed after 72 h in the broth grown at salinities of 0.5% with a clearing zone of 16 mm. (Figure 3).

Antagonistic action of *B. licheniformis* at the optimized conditions: Production of the antagonistic component was observed at the optimized conditions (i.e. pH 7.5, 0.5% salinity and at 30°C) right after the 24 hours of growth, which attained the peak after 72 hours post-harvest when the culture entered the late log phase, and the activity peaked in the stationary phase and remained stable thereafter, with only minor fluctuations in the activity (Figure 4).

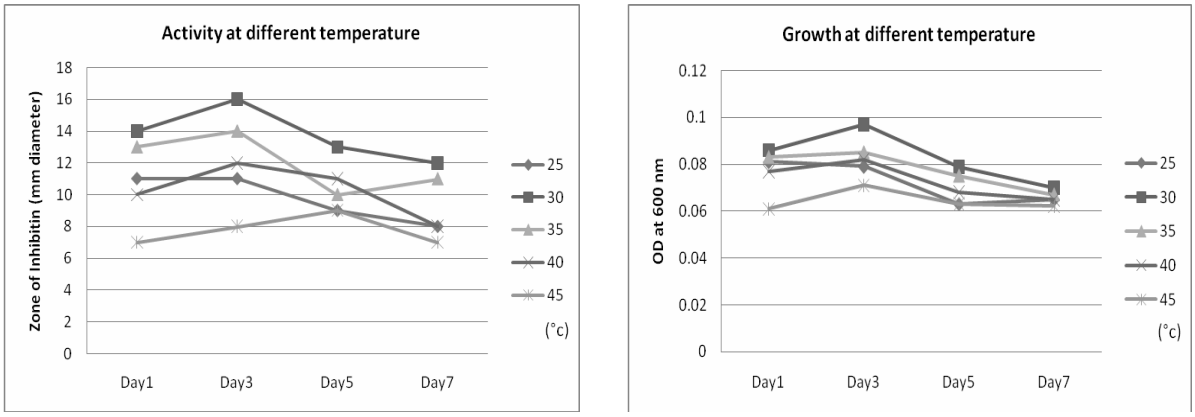

Figure 1. Optimum temperature for growth and antagonistic action of the probiont. *B. licheniformis* was harvested in nutrient broth at temperatures, 25, 30, 35, 40 and 45°C; and culture supernatants were collected at days 1, 3, 5 and 7 post-harvest. The vibriocidal activity of *B. licheniformis* was tested in petridishes containing 50 µl of cell-free extracts, absorbed in empty discs. The zone of inhibition produced around the discs against the growth of *Vibrio* sp., previously swabbed in the plate, after 48-hour incubation at 30°C is plotted in X-axis. A disc absorbed with the medium only was used as negative control that produced no inhibitory zone (A). The respective growth of *B. licheniformis* at different temperatures and at different time intervals was measured by taking absorbance at 600 nm (B).

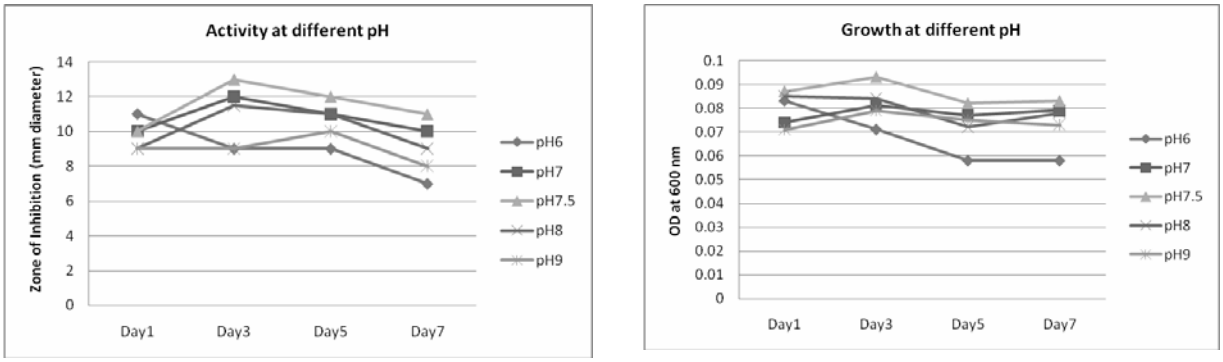

Figure 2. Optimum pH for growth and antagonistic action of the probiont. *B. licheniformis* was harvested in nutrient broth at pH, 6.0, 7.0, 7.5, 8.0 and 9.0; and culture supernatants were collected at days 1, 3, 5 and 7 post-harvest. The vibriocidal activity of *B. licheniformis* was tested by disc-diffusion method and is plotted in X-axis (A). The respective growth of *B. licheniformis* at different pH and at different time intervals was measured by taking absorbance at 600 nm (B).

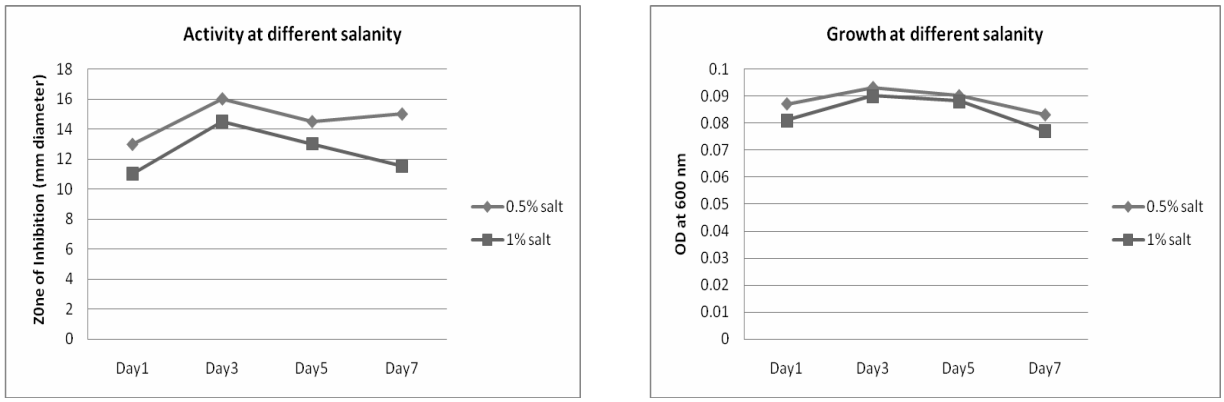

Figure 3. Optimum salinity for growth and antagonistic action of the probiont. *B. licheniformis* was harvested in nutrient broth at 0.5 and 1.0% salinitities; and culture supernatants were collected at days 1, 3, 5 and 7 post-harvest. Its vibriocidal activity was tested by disc-diffusion method and is plotted in X-axis (A). The respective growth of *B. licheniformis* at different salinities and at different time intervals was measured by taking absorbance at 600 nm (B).

Partial characterization of the antibacterial component in cell-free supernatant of *B. licheniformis*: The cell-free supernatant was treated with Triton X114 (10%, 20% & 30%), Tween 80 (10%, 20% & 30%), SDS (0.1 mM, 0.05 mM & 0.01 mM), EDTA (0.1 mM, 0.05 mM & 0.01 mM). Then antibacterial activity against the target *Vibrio* was tested by the disc diffusion assay. While the extract alone produced a 7 mm zone of inhibition, the detergents- Triton X114, Tween 80 or SDS at their different concentrations also produced greater zones of inhibition around the discs even in absence of the extract, used as controls (Figure 5). Hence, their effects were refractory to the antibacterial component of the probiont. Treatment with the metal-chelating substance, EDTA however, nullified the antibacterial activity of *B. licheniformis* completely, at its 0.01 mM concentration; the lowest tested, a strength that does not produce any vibriocidal effect on its own; thereby indicating the requirement of a metal active group in the active fraction of the probiont's cell-free supernatant.

The current concern over the spread of antibiotic resistance genes due to indiscriminate use of antibiotics in the aquaculture industry, the failure to identify new antibiotics and the inherent problems with developing new vaccines make a compelling case for developing alternative prophylactics. The potential use of probiotics could open new avenue in combating this problem. In fact, the field of probiosis has emerged as a new science with applications in farming and aquaculture as alternatives to

antibiotics.[2] To date, no study had been conducted in controlling bacterial infection in shrimps on farms in relation to probiotic usage in Bangladesh. A good number of probiotics, purchased from foreign origin, however were tested in some hatcheries and culture ponds of Khulna region, and was not found effective (personal communication). This observation therefore demands to discover new probiotics from the indigenous origin to be effective in local environment.

The available literature shows that it is possible to change bacterial species composition in large water bodies, hatchery tanks and prawn guts and improve prawn production.[2] *Vibrio* species can be controlled in this manner. The control of virus disease is possible, but is more complex than controlling pathogenic bacteria. Fish and shrimp farmers who manage the microbial ecology of their ponds are succeeding in the presence of white spot virus and *Vibrio*.

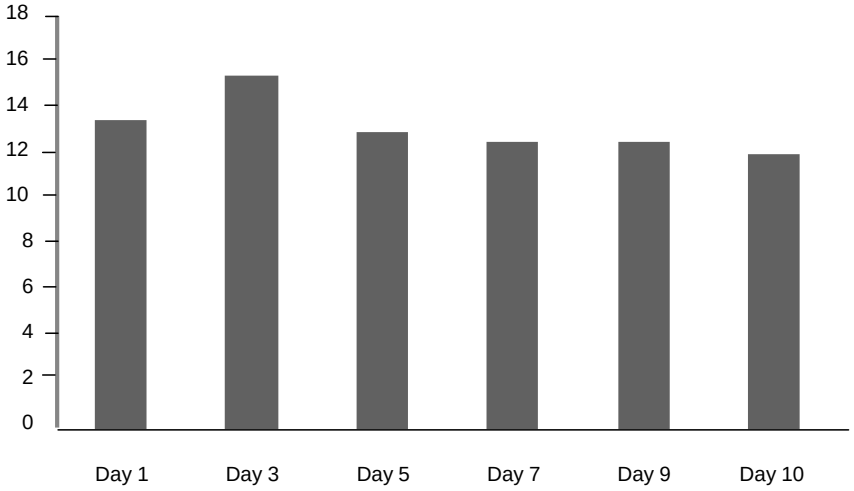

Figure 4. Optimization of the antagonistic action of the probiont. *B. licheniformis* was harvested at optimized conditions (30°C, pH 7.5 and at 0.5% salinity) for ten days. The culture was withdrawn as indicated time intervals to assess the vibriocidal activities of the respective cell-free extract by disc-diffusion assay.

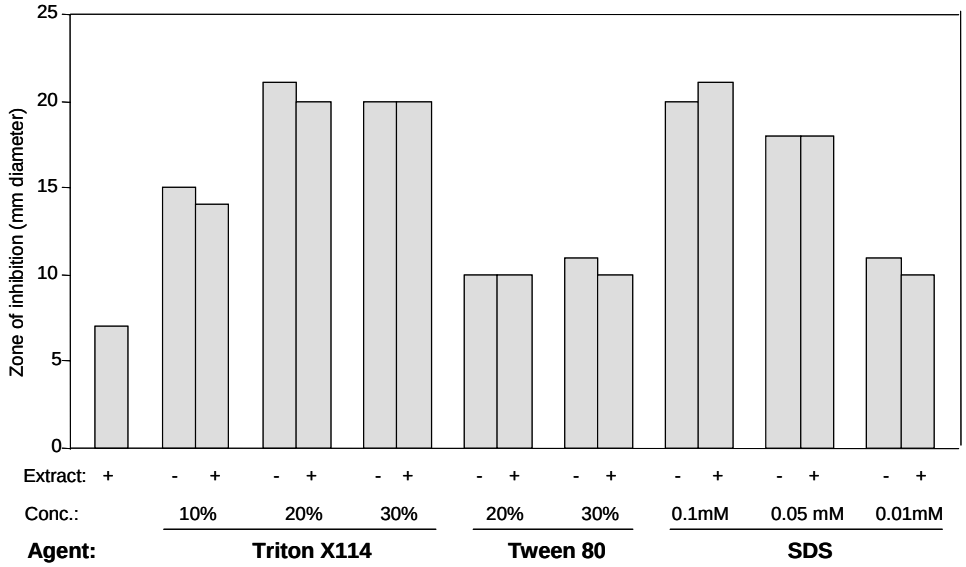

Figure 5. Partial characterization of the antagonistic action of cell-free supernatant (Extract) of *B. licheniformis.* Effects of detergents, viz. Triton X114, Tween 80, and SDS at their different concentrations (as indicated) are mixed with (+) or without (-) the extract of the probiont (Extract) in discs and then tested on the vibriocidal activity by disc-diffusion assay on a agar plate previously swabbed with the pathogenic *Vibrio* spp. Results are plotted in terms of the zone of inhibition produced around the discs.

The results in this study show that *B. licheniformis* isolated from the rearing environment of shrimp aquaculture produced a wide zone of inhibition against several *Vibrio* sp. tested. The result of disc-diffusion method indicates that it is not the bacterial cell but an extracellular product that is likely to be responsible for inhibition. Likewise, Vijayan *et al* (2006)[14] noted that a cell-free culture supernatant of a brackishwater isolate, *Pseudomonas* appeared as a potential antagonistic bacterium against pathogenic vibrios in penaeid and non-penaeid rearing systems. It can be suggested that *B. licheniformis* produces some extracellular anti-vibrio component. These results are very significant since *V. harveyi* is a great problem in shrimp hatcheries. One of the most important criteria for a candidate to be used in biocontrol is that the organism should be nonpathogenic to the host. Characterization of the inhibitory component in the cell-free extract of *B. licheniformis* suggests that it is a metal active group-requiring extracellular substance that exhibit vibriocidal activity.

The maximum production of antivibrio factor was observed at pH 7.5 (Figure 2) and at temperature of 30–37^{o}C (Figure 1) which was also the optimum pH and temperature for the growth of the organism. This suggests a close relation between growth of *B. licheniformis* and production of antivibrio factor. This is further confirmed by results in Figure 3, which show that production of antivibrio factor was the highest at a NaCl concentration of 0.5% which was optimal for growth of the organism. The appearance of the antivibrio activity in the medium when the cells reach stationary phase of growth and maximum activity at late stationary phase (Figures 1-3) suggests that the antivibrio factor is a secondary metabolite. This activity of the isolate was found evident in a wide range of environmental tolerance: temperatures from 25^{o}C to 40^{o}C, pH from 6 to 8, and salinity from 0.5% to 1%. Further, when the isolate was grown in the optimized environmental conditions, i.e. at 30^{o}C, pH 7.5 and at 0.5% salinity, the antagonistic activity rose at its peak in 3 days, and remained significantly active even at the 10th day of post-harvest (Figure 4). In conclusion, it can be stated that the isolate of *B. licheniformis* has the properties of a biocontrol agent for use in shrimp hatcheries and farms.

Probiotic treatment offers a promising alternative to the antibiotics for fish and shrimp aquaculture system. Since probiotics are made from natural mechanisms, shrimp farmers who learn to farm microorganisms will be far more likely to achieve successful harvests. Therefore, the probiotic bacteria, isolated from this study might have the potentiality to control the shrimp larval pathogens and may substitute the use of antibiotics in aquaculture, once it successfully passes the safety tests.

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