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Incidence of Antagonistic *Bacillus* Spp. - An Eco-friendly Aquatic Probiotic from aquaculture Ponds

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Abstract

Specific, non-pathogenic, pigmented bacterial species of Bacillus isolated from sediments of pokkali and perennial ponds of Narakkal are found to inhibit growth of pathogenic bacteria like *Vibrio, Aeromonas, E. coli* by competitive growth or by producing antagonistic antibiotics. Gram-negative slime-forming myxobacteriaceae, an undesirable organism in aquaculture ponds are also inhibited by two Bacillus spp. and can be included in detritus management system of bacteria showing antagonistic activity. Totally 7 species of Bacillus were isolated from sediments of aquaculture ponds during the period of study from March '95 to February '96 out of which pink pigmented Bacillus dominated and formed 12% of total heterotrophs. The results suggest that Bacillus spp.can be successfully used as probiotics in feed preparations and for management of detritus in intensive aquaculture operations to control the attack of microbial diseases reducing high shrimp mortality.

Introduction

It has been estimated that 75 to 80% of shrimp disease occurrence is due, at least partly to stress and environmental factors and therefore potentially avoidable. The importance of environmental factors has been evident in the national and regional variation in bacterial and viral disease incidence and in mass mortality (Austin 1998). The changes in disease occurrence rates are most important in analytical epidemiological studies. According to Kinne (1980), a disease may result from the effect of pathogens, pollution, physical injury, nutritional imbalance and genetic disorders. Antagonistic *Bacillus* spp. isolated from sediments can be incorporated in feed or included in the detritus management system (DMS) of ponds to enhance the cell-mediated immunity of crustaceans.

Materials and Methods

With a view to find out seasonal distribution of heterotrophs in sediments of aquaculture ponds, samples were collected from perennial and seasonal ponds of Narackal from March 95 to February 96 during which period *Bacillus* spp. was the main candidate of sediments samples.

The pond sediment samples were first partially dried at room temperature to induce sporulation of vegetative cells of spore-forming bacteria. Counts obtained by heating were less when compared to counts obtained after drying. 1 gram of sediment rich in organic matter was partially dried in sterile weighing bottles. A suspension was prepared by dilution and 3-5 ml were transferred to small sterile tubes which were placed in a rack in a water bath set at 80°c for 10 minutes.

Inoculation from this pasteurized suspension was made by serial dilution and by pour-plate method. The culture medium used for spore-forming species was a mixture of beef-peptone and seawater agar combined in a proportion of 1:1. The pH was maintained at 7.0 to 7.2. The plates were incubated at 25-30°c for 48 to 72 hrs. Different species of spore formers yielded characteristic colonies on the medium used. A rough estimate of the number of bacteria was made on the basis of morphology of the colonies. The plates were retained for 4-30 days at room temperature (RT) after estimating the colony forming units (C.F.U.).

Results and Discussion

Colonies of spore-formers most often observed were identified as the following 7 species.

Bacillus cereus: Sub sp. *Mycoides* (flige) Smith *et al.*, (1952) spreading over the surface with curving filaments radiating out from the central growth and are easily differentiated by their feathery appearance. The growth may be dry, gummy or moist white, greyish white, yellowish or brown. Certain species produced black pigment also.

Bacillus megaterium (Bary): Smooth white butyrus, shiny colonies consisting of typical Gram-positive rod-shaped cells,

Table 1. Biochemical characteristics of Bacillus strains

Test	Result	% Positive
Gram-stain	Gram+long rods	100%
Sproes	Round & Oval types	100%
Nitrate reduction	+	79.6%
HUGH & Leifson's (a) Oxidative	+	33.0
(b) Fermentative	+	66.0
Citrate Utilisation (Christensen)	+	88.0
Urease (Christensen)	-	44.4
Indole	-	-
Voges-Proskauer	+	76.0
Gelatin liquifaction	+	95.0
Casein hydrolysis	+	100.0
Starch hydrolysis	+	83.0
Acid from Glucose	+	100.0
Lactose	-	8.0
Sucrose	+	55.0
Manitol	-	60.0
Maltose	+	12.0

classical representative group in the genera of *Bacillus*.These are the *Bacillus subtillis* group.

Bacillus mucosis: (Zimmerman) - mucoid semitransparent resembling drops of pastes.

Bacillus agglomeratus (migula) small greyish round colonies.

Bacillus cartilaginous: (Krasilinikov) - Thick, round compact colonies, which can be lifted from the agar in their entirety.

Bacillus idiosus (Burchard): Dry lusterless colonies, laminated, finely wrinkled.

Bacillus intrieatus (migula): Widely spreading whitish colonies, flat, mycelium like in growing into the agar, containing filaments with numerous septa.

All the 7 species were collected from sediments of seasonal ponds (Pokkali field) and perennial ponds during 1995-96 out of which pink pigmented *Bacillus* with central spores dominated and formed 12% of total heterotrophs.

24 random colonies with different morphological appearance (Table 2) were isolated and maintained in the slants after primary isolation on sediment extract agar. Some strains were also isolated from primary cultures on Andersens seawater agar (Andersen 1962).

The criteria for identification of strains were the presence of a spore and ability to grow aerobically in room temperature. Gram-Stained smears were observed after 24-30 hours of growth on sediment extract agar or soyabean agar. Spore-stain was done and clear distinction was made between swelling and non-swelling round and oval types of spores appeared in the same preparation.

Hilen (1923) recorded seven species of *Bacillus* from fouled surfaces indicating their predominance in marine environment., Venkataraman and Sreenivasan (1955b) found nine *Bacillus*, some pigmented from marine environment and from fresh shark. Wood (1940) encountered 10% *Bacillus* in spoiling fish from market but in trawled fish only 9% was isolated. In the present investigation 7 species of *Bacillus* including pink *Bacillus* with central spores formed 12% of the total heterotrophs. Venkataraman and Sreenivasan (1955b) encountered 40.3% of *Bacillus* from Calicut seawater out of the 72 heterotrophs isolated and 50.8% from mackerel out of 65 colonies. Wood (1940) isolated 37% from gut of fishes 12% from sea water and market air, 9% from gills and slime of fish and 8% from tap water.

Table 2. Morphological diversity of micro-organisms in perennial and Pokkali field

Category of Micro-organisms	% of Total Isolates	
	Perennial Ponds	Pokkali Field
Rods, non spore forming Gram-negative	18-22	18-30
Gram-positive	4-6	3-9
Rods, spore forming	10-15	14-20
Pleomorphic bacteria	32-48	29-46
Actinomycetes	10-24	11-34

Biochemical characteristics

Spore forming rods, motile, forming chainlets. The spores were oval cylindrical and not strictly localised. Gram positive, growth in fluid media was classified as

a. Clearing with sediment

b. Uniform turbidity

In seawater agar, with 1% glucose growth occurred as a wrinkled whitish film. There was dense precipitation of grey-black poly, metasulphide particles, which generate smoke-like plumes.

Gelatin was liquefied in six days and 95% of *Bacillus* exhibited proteolytic activity. 100% casein hydrolysis was recorded among these isolates.

Hugh and Leifsons test showed that 33% were capable of oxidatively fermenting glucose and 66% were fermentatively utilising glucose in the absence of oxygen (Table 1). All 24 strains tested utilised glucose. Only two strains utilised lactose and 3 strains utilised maltose. Sucrose and mannitol were fermented in 6 days and all strains fermented the sugars after the 8th day. 83.3% isolates hydrolysed starch in 24 hours incubation time. Urease activity was recorded in 44.4% of the total. Indole production was completely absent but citrate was utilised by 88% of the isolates and 95% were positive for Voges-Proskauer reaction. 79.6% reduced nitrate. Growth was found satisfactory in 5% and 10% sodium chloride.

Antagonistic activity

Antagonistic activity of the 7 *Bacillus* was tested against identified test pathogens like *Vibrio anguillarum*-H^{10} obtained from National Collection of Type culture, Central Public Health Laboratory, London, *Aeromonas slamonicida, Escherichia coli* and *Cytophagha psychrophila* by cross-streak assay.

The zone of inhibition (diameter) was measured in mm after the incubation period - 24-72 h in sea water agar. All the cultures tested were able to inhibit the growth of *Aeromonas salmonicida, E. coli* and *Cytophaga psychrophila* and only 4 strains exhibited activity against *Vibrio angullarum*H^{10}. Sea water agar was the medium used for the test as it was found best for isolation, maintenance and also to test the anti-microbial activity in the present study. Each strain was maintained on seawater agar slants, sub-culturing every fortnight till the bio-chemical and anti-microbial activity studies were over.

Isolates showing inhibition zones of 10-12 mm against the test pathogens were treated as sensitive, (Table 3) eventhough all the 7 *Bacillus* exhibited 4-6 mm inhibition zone to all the 4 test pathogens.

Table 3. Antibiogram of *Bacillus* strains isolated from Perennial and Pokkali field by cross-streak-assay method

Antagonistic *Bacillus*	*V. anguillarum* Zone of inhibition in mm			*Aeromonas salmonicida* Zone inhibition in mm			*Escherichia coli* Zone inhibition in mm			*Cytophaga prychrophila* Zone inhibition in mm		
	4-6	7-9	10-12	4-6	7-9	10-12	4-6	7-9	10-12	4-6	7-9	10-12
B. creus sub. mycoidas			+			+			+			+
B. megaterium			+			+			+			+
B. mucosis	-			+			+					+
B. agglomeratus	+			+			+					+
B. cartilaginous	+			+			+			+		
B. idious	_			+			+			+		
B. intrieatus	-			+			+			+		

Beneficial, eco-friendly bacteria are a must for healthy prawn culture. However water treatment, with chlorine, iodophores and antibiotics kill the beneficial autochthonous microbes as well as pathogenic allochthonus microbes, reducing fertility of the water. It was the practice, that the only way to combat diseases was to apply Tetracyline-a 'cure all' for all diseases. Bio-augmentation with imported detritus management system (DMS) bacterial cultures is difficult and expensive because of the low concentration of bacteria in these products. 10^{-9}/gm of DMS - *Bacillus* has to be added to mineralize and reduce the faecal matter of animals and left over feed in intensive aquaculture so that there will be an increase in survival and growth rate of animals and reduction in the bottom sludge. Breakdown of organic matter will stimulate phytoplankton and zooplankton growth and H_2S, NH_3 and CH_4 will be periodically removed successfully. Apart from use in detritus management these antagonistic *Bacillus* spp. can be incorporated in fish feed as probiotic.

The present investigation on *Bacillus* was motivated by the increasing importance of this genus in prawn farming bio-industry and to public health. In non-selective media for coliform counts *Bacillus* was found inhibiting the growth of these coliforms by formation of antibiotics or by competitive growth (Bonde, 1968). Being ubiquitous, these spore-bearers have attracted great interest in works on ecology and may indicate pollution. Zobell and Upham (1944) isolated several species of the genus *Bacillus* from the marine mud around pacific coast. Rod, shaped *Bacillus* are heat resistant spore-bearing, usually gram-positive, catalase producing come under order Eubacteriales and family Bacillaceae frequently isolated from sediments in the present investigation. 24 isolates of marine *Bacillus* - the indicator of refractory organic compounds from different sediment samples were studied for their biochemical and physiological characters and for the effect of incubation temperature on the growth of *Bacillus subtilis* injured spores. 36 *Bacillus* from sediments of Cochin Backwater showed high proteolytic, ureolytic and caseinolytic activity (Chandrika 1983).

Colwell and Liston (1960) isolated only 6% *Bacillus* from Chesapeake Bay out of 229 heterotrophs isolated which showed poor representation of this genera in the Bay. Only 4.6% *Bacillus* was isolated from oysters from controlled and natural environments *i.e.*, 7 strains turned out to be *Bacillus* out of 152 cultures isolated from the oysters caught from pacific coasts. Litchfield and Wood (1966) found that by proteolytic activity of *Bacillus* higher enzyme yields was obtained when protein was suspended in medium than when it was dissolved in the medium. Buck (1976) isolated 7% *Bacillus* from Connecticut River in the prethermal and thermal discharge periods. From Lake Macquarie, Wood (1959) isolated proteolytic *Bacillus* from surface water and bottom sediments of 1 metre depth; the genus *Bacillus* predominated and formed 22% from surface water 39.5% from 1 m and 45% from bottom sediments. New species producing specific enzymes are being studied with reclassification of certain species and variants, for use as probiotics and DMS - range of bacteria to enhance intensive shrimp/fish culture.

In the present study 7 *Bacillus* species showed antagonism, proteolytic, and catalytic activity and can be used for bioremediation in aquaculture pond. Bioremediation is the use of entire DMS - range of micro-organisms of enzymes to transform a toxic substance to benign or less toxic or preferably to Co_2 and water. This require adaptation of micro-organisms that can oxidise, modify or break down toxic materials by their ability to use them as carbon or nitrogen sources. The development of eco-friendly *Bacillus* which is non-pathogenic and analyticaly convert wastes by bio-process technology to harness faecal wastes and left over feed of animals in ponds will enhance the productivity of the pond. By reducing the stress due to wastes, the immune system of crustaceans may be enhanced as it is not well developed especially in shrimps. The dominance of *Vibrio* sp. in culture system and reared shrimps after 60 days culture is of great concern, (Jawahar *et al.*, 1996). Most of the species of genus *Vibrio* have been shown to be primary pathogens or secondary pathogens to shrimps (Lightner 1993). Epidemics from adjoining pond will not spread if DMS - range antagonistic *Bacillus* are added every fort night, as natural resistance will be maintained by improving water quality. The deliberate release of microbes in the pond will not create any imbalance in the environment as it is biodegradable, leaving no residues. Intensive farming has resulted in spread of diseases which can be controlled by reducing the stress of the animal by waste minimisation and by increased resistance of animals. Enzymes from bacteria and fungi are being currently developed for such purpose and antagonistic *Bacillus* isolated in the present study is a suitable candidate for feed preparation with probiotics and can be included in DMS - range of bacteria for use in intensive aquaculture operations.

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