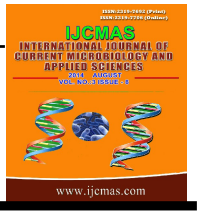
Int.J.Curr.Microbiol.App.Sci (2014) 3(8) 474-480

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 8 (2014) pp. 474-480**
http://www.ijcmas.com



Original Research Article

Antimicrobial activity of *Bacillus cereus* strain isolated from Rohu (*Labeo rohita)*

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A B S T R A C T

Five species of Bacillus bacteria were isolated from the intestine micro flora of Rohu (*Labeo rohita*) to evaluate the probiotic properties. *Bacillus cereus* with more ability to inhibit the growth of *Aeromonas hydrophila* was selected and identified by conventional and biochemical tests. This bacterium also, showed antimicrobial activity against fish pathogen *A. hydrophila.* Out of the five isolated bacterial strains, only one strain of *Bacillus sp* was identified as efficient. Morphological characteristics of *Bacillus sp.* revealed that it is a Gram positive, rod shaped and motile. Biochemical results were positive for voges-proskauer, catalase, starch, gelatin, citrate utilization, nitrate reduction, spore formation, glucose, fructose, maltose, sucrose, ribose and trehalose; whereas it showed negative results for oxidase, indole, mannitol, xylose, lactose and sorbitol. The present study concluded that *B.cereus* was clearly beneficial for culturing *Labeo rohita* when administered as a food additive. It is argued that such probiotic has a role in disease control strategies, growth promotion and immune stimulation.

Keywords

Probiotic, *Labeo rohita*, Antimicrobial activity, *Bacillus cereus*, *A. hydrophila*

Introduction

Culture of Indian major carps (IMC) contributes more than 80% of the total aquaculture production in India. *Labeo rohita* (rohu) with its high consumer preference and good growth rate, it is widely cultured in Indian subcontinent (CIFA, 2004; FAO, 2005; Ayyappan and jena, 2001). Rohu (*Labeo rohita*) is widespread and popular fish in Southeast Asia. This fresh water fish has different beneficial effects, and used as wound healing and remedies (Jais *et al.*, 2002).

Aquaculture has been increasing in recent decades as a consequence of the increase of fish consumption, since fisheries have possibly reached their maximum due to overexploitation (FAO, 2003; Ferreira *et al.*, 2008). It is now accounting for almost 50% of the world's food fish (FAO, 2006; Martins *et al.*, 2011). Globally, aquaculture is expanding into new directions, intensifying and diversifying (Denev *et al.*, 2009). There has been a phenomenal shift from extensive to intensive culture of carps in the last three decades.

Intensive aquaculture offers an increased opportunity for spreading of infectious diseases at all stages of production (Bondad-Reantaso *et al.,* 2005). Among the bacterial pathogens *Aeromonas hydrophila* is a ubiquitous secondary pathogen of IMC including rohu. Several instances of infections with *A. hydrophila* in India have been reported in IMC in recent past (Shome *et al.,* 2005) hence; vaccination of aquacultured fish is becoming inevitable with increasing health risks.

Probiotics application in aquaculture sector is gaining importance, with the development of eco-friendly aquacultural practices (Gatesoupe, 1999). Probiotic may be defined as a live microorganism that offers a beneficial effect in the host when ingested in required amounts (FAO, 2001). In aquaculture, administration of probiotics can be done through dietary supplement or as a water additive (Moriarity, 1998). 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 (Moriarity, 1997). Moreover, they cause a deleterious effect on the environment (Bachere, 2000; Bachere, 2003). Hence an effective alternative for the usage of antibiotics could be the application of probiotic which would favour the environment (Balcazar *et al.,* 2006; Verchuere *et al.,* 2000).

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. (Verchuere *et al.,* 2000; Thompson *et al.,* 1999). In fish and shellfish culture, photosynthetic bacteria, yeast, *Bacillus sp. and Lactobacillus sp.* have been evaluated (Gatesoupe, 1999; Vine *et al.,* 2006; Kesarcodi-Watson *et al.,* 2008). Bacillus had been extensively used as efficient probiotics (Ziaei-Nejad, 2004) due to the capability that they secrete a wide range of exoenzymes and antimicrobial compounds (Moriarity, 1998: Moriarity, 1996). 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 (Robertson *et al.,* 2000). 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 Major Carp *Labeo rohita*, the present investigation was carried out.

Materials and Methods

Fish and Sampling

Labeo rohita (Rohu) were acclimatized in the laboratory in plastic tubs (150 L) filled with fresh water and continuous aeration. During this period, fish were fed with control diets at 2% of body weight. Water was replenished to the extent of 40-50% on alternate day. Fresh fish were randomly selected then the total body weight and total length measured. The surface of fish bodies were disinfected by alcohol (70%); dissected under antiseptic conditions; intestines taken out and washed three times with normal saline (NaCl 0.85 % w/v). The intestines

were then cut in small pieces (1 g) and homogenized (Rengpipat *et al.*, 2008).

Isolation of Fish Gut Bacteria

Using serial dilution (up to 10-4 CFU/ml, NS), 0.1 ml of homogenized intestine samples was spread on tryptic soy agar (TSA) (Merck) followed by 48 h incubation at 37°C to count total colony of bacteria (Paludan-Mu¨ller *et al.*, 1999). The intestine samples were then immersed in de Man Rogosa and Sharp (MRS) (Merck) broth and incubated at 37°C for 24 h. After pipetting, 0.1 ml of the cultured broth was transferred to MRS agar containing bromo-cresol purple (0.17 g/L) (Badis *et al.*, 2004; Rengpipat *et al.*, 2008). The plates were incubated at 37°C for 48 h under anaerobic condition. The colonies sub-cultured three times on new MRS agar to obtain single colonies (Rengpipat *et al.*, 2008). Gram staining, catalase reaction by 3% hydrogen peroxide and also morphology using phase contrast microscopy were used for primary identification of the isolates (Nguyen *et al.*, 2007; Kopermsub and Yunchalard, 2010).

Identification of biochemical test in probiotic bacteria

All isolates were sub-cultured and gram-staining was carried out. Identification of isolates was carried out based on the method described by Sakazaki and Shimad (1986), Collins *et al.* (1989) and Cheesbrough (2002). The gram staining was aimed at differentiating gram reactions, sizes, shapes and arrangement of cells of the isolates. Based on Bergey's Manual (Al-Ajlani and Hasnain, 2010), the strains were classified using the following criteria: Morphology, Gram reaction, sporeformation, Motility, Indole, Oxidase, catalase activity, Citrate, utilization, lecitinase activity, production of gas from glucose, sugar fermentation (D-glucose, D-mannitol, lactose, sucrose, melibiose, D-xylose, L-arabinose, maltose and salicin, 1 g/100 mL), reduction of nitrate, and gelatin and starch hydrolysis.

Source, Isolation and Maintennance of *Aeromonas hydrophila*

Antibacterial activity of the strains was tested by disc and well diffusion techniques using cell-free cultured broth of the individual selected colonies (Cappuccino and Sherman, 2002). Briefly, *Aeromonas hydrophila* as freshwater fish pathogen was cultured in TSB broth; incubated at 37°C for 24 h; and then streaked on TSA plates. At the same time, the isolates were cultured in MRS broth for 24 h at 37°C; the bacterial cells were precipitated at 8000 rpm and 4°C for 5 min; the supernatant was used for the assay. Sterile disc were immersed in the supernatant; air dried; and placed on TSA plates containing *A. hydrophila*. The plates were incubated at 37°C for 24 to 48 h to observe inhibition zone (Aly *et al.*, 2008; Lauzon *et al.*, 2008; Rengpipat *et al.*, 2008). 1 of the isolates with the greatest inhibition zone was selected for more potential probiotic evaluation.

Results and Discussion

Out of the five isolated bacterial strains, only one strain was efficient in terms of satisfying the probiotic characters *i.e.* in terms of extracellular enzymes production (protease, amylase, lipase and phytase). This efficient probiotic bacterium was identified as Bacillus *sp.* through conventional method *viz.* Morphological and biochemical characteristics. Morphological characteristics of Bacillus *sp.* revealed that it is a Gram positive, rod shaped and motile. Biochemical results were positive for voges-proskauer, catalase, starch, gelatin, citrate utilization, nitrate reduction, spore

formation, glucose, fructose, maltose, sucrose, ribose and trehalose; whereas it showed negative results for oxidase, indole, mannitol, xylose, lactose and sorbitol (Table 1).

Bacillus strains are widely used as probiotics, because they are capable of spore formation and are much resistant to physical and chemical effects, and thus determines their longevity in the environment (Henriques and Moran, 2000; Nicholson *et al.,* 2000). Usually *Bacillus* strains are able to produce exoenzymes (Moriarity, 1997) and largely they are associated in the marine environment *e.g., Bacillus* bacteria isolated from crustacean intestines (Nicholson *et al.,* 2000) and bivalves (Sugita *et al.,* 1981). In the present study, a candidate probiotic bacterium *B. cereus* was isolated from the gut of IMC *Labeo rohita*, that showed marked antagonistic activity against fish pathogen *Aeromonas hydrophila*. This result is in agreement with the findings of Sugita *et al.* (1998) and Rengpipat *et al.* (1998), who reported that species of genus Bacillus showed inhibitory activity against various fish pathogens. Probiotic bacteria produce digestive enzymes and required growth nutrients such as vitamins and amino acids thereby improving the feed absorption resulting in an enhanced growth rate in host.

Table.1 Biochemical characters of *Bacillus cereus* and *Aeromonas Hydrophila*

Biochemical tests	*Bacillus cereus*	*Aeromonas hydrophila*
Morphology	Rod	Rod
Gram staining	+	-
Motility	+	+
Spore formation	+	+
Citrate utilization	+	+
Catalase	+	+
Galatin hydrolysis	+	+
Starch hydrolysis	+	-
Indole	-	+
Oxidase	-	+
Voges-proskauer	+	-
Nitrate reduction	+	+
Glucose	+	+
Fructose	+	-
Ribose	+	-
Maltose	+	-
Sorbitol	-	+
Mannital	-	+
Sucrose	+	-
Lactose	-	-
Xylose	-	-
Trehalose	+	-

In general, results obtained from antibacterial test indicated that well diffusion method was more effective than disc diffusion method. Aly *et al.* (2008) reported that the growth of *A. hydrophila* was inhibited by three species of *Bacillus* bacteria that used as probiotic and also, Rengpipat *et al.* (2008) confirmed growth inhibition on *A. hydrophila* using a cell-free cultured broth of *Bacillus* bacteria. Kim and Austin (2008) determined the antibacterial ability of two probiotic strains that were isolated from rainbow trout intestine against *A. hydrophila* and *A. salmonicida*. These strains inhibited the growth of both *A. Hydrophila* and *A. salmonicida.*

Moreover, similar results for *Lactobacillus* delbrueckii were reported by Pan *et al.* (2008). Since intestinal tract and faeces can serve as an enrichment site for pathogenic bacteria such as *Aeromonas* and *Vibrio* species, the use of probiotics with antagonistic activity may be used to reduce or inhibit pathogens activities (Balcázar *et al.,* 2008). In general, *Bacillus* species have different ability to inhibit growth of pathogenic bacteria. Therefore, the findings in this study suggest that *Bacillus cereus* may have high potential probiotic and anti adhesion effect against pathogens.

Acknowledgement

The author is thankful to the Principal, Periyar E.V.R. College and Head of the Department of Zoology, Tiruchirappalli for extending their support in carrying out the research work.

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