Probiotic assessment of Bacillus infantis isolated from gastrointestinal tract of *Labeo rohita*

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Abstract- The present study was aimed to isolate, characterize and examine the probiotic properties of *Bacillus infantis* (KADR2) from *Labeo rohita*. The total of seven strains isolated from gastrointestinal tract of *Labeo rohita*, one of them KADR2 showed higher antagonistic effect against fish pathogens. The strain was evaluated under *in vitro* intestinal condition based on resistance to bile tolerance, low pH, hydrophobicity, catalase activity and antibiotics susceptibilities. Partial 16S rRNA gene sequence of this strain KADR2 were blasted and showed homology with *Bacillus infantis*(99%) supported by morphological and physiological characterization. Consequently, the positive results of this study suggested that further studies in challenge experiments in fish to explore their probiotic effects having great scope for being used as a potential probiotic in aquaculture.

Index Terms- Aquaculture, Fish pathogens, *Labeo rohita*, Probiotics

I. INTRODUCTION

Aquaculture is emerging as a major enterprise supplementing the needs of animal protein demand for human. Fish are associated with several harmful pathogens produced the emergence of infectious diseases caused by bacteria, fungi, virus, protozoa and parasites present in the aquatic environment. Bacterial diseases are responsible for severe economic losses and high mortality in aquaculture industries (Wang et al. 2008). However, the continuous use of antibiotics in order to manage pathogenic microorganisms results in causing major changes in the normal microbiota in and around the aquaculture systems, increasing resistance to common antimicrobials (He et al. 2010, 2011, 2012; Resende et al. 2012). *Labeo rohita, Catla catla and Cirrhinus mirgala* are the major carp's production of Indian aquaculture. This carps are frequently affected by *Aeromonas hydrophila* infection that associated with tail and fin rot, hemorrhagic septicemia (MAS) and epizootic ulcerative syndrome (Vivas et al. 2004). This infection decreases the yield to fish cultivators. Hence, it is need of the hour to find and defend harmful pathogens with alternative methods. Probiotics are live cell preparations having beneficial features like improving its feed value, enzymatic contribution to digestion, inhibition of pathogenic microorganism's growth promoting factors and an increased immune response to the host (Verschuere et al. 2000).

The research on the live cell preparations in aquatic organisms is being increased to sustain the aquaculture industry. The *Lactobacillus* spp., *Bacillus* spp., *Saccharomyces cerevisiae*, and *Lactococcus* spp. are the commonly used probiotics in carps (Ramakrishnan et al. 2008; Harikrishnan et al. 2010; Geng et al. 2012). *Bacillus* species are economically and industrially important strains compare to others, because they produce endospores that tolerant to heat and longer shelf life to produce diverse amount of secondary metabolites (Jock et al. 2002; Puniya et al. 2012). Potential probiotic microorganisms one of the alternatives to antibiotic and chemotherapeutic agents in disease control to sustainable aquaculture production and eco-friendly. Therefore, the aim of this study was to isolate and to select the most promising *Bacillus* strains from fish gut. .

II. RESEARCH ELABORATIONS

Materials and Methods

Isolation and identification

Indian major carp, *Labeo rohita*, (Hamilton) (with average weight >20g) were collected from Cauvery River, Tiruchirappalli District, Tamil Nadu, India and brought alive to the laboratory. Ventral surface sterilization was done using double distilled water followed by 70% ethanol. Under sterile conditions, the fish gut region was dissected out and homogenized with 5 ml of normal saline. The homogenate was kept in a boiling water bath at 80 ºC for 20 min and kept in normal tap water immediately. The homogenate was used as inoculums which was serially diluted and plated on *Bacillus* agar medium and incubated at 37 ºC for 24 h. Single isolated colonies were picked and purified on another *Bacillus* agar medium. The purified isolates were tested for gram staining, spore staining, catalase activity, oxidase activity, MR-VP, indole production, citrate utilization and carbohydrate fermentation. The nucleic acids of each strain were extracted from the 12 h culture following the phenol-chloroform isolation method (Joseph Sambrook 2001). Universal primers 27F (5' CCAGAATTCAGAGTTTGATCMTGGCTCA3'), 1492R (5'ACCAAGCTTTACGGYTACCTTGTTAGGACTT-3') were used to amplify 16S rRNA gene sequence of the isolates. The PCR reactions were carried out in a total volume of 50µl (consisting of 5 units Taq DNA polymerase, 400mM each dNTPs, 1.5 mM $MgCl_2$ and 20ng template DNA). PCR was performed in a Thermal cycler (Eppendorf) under the following condition 95 ºC (5min), followed by 34 cycles of 94 ºC (1 min), 58 ºC (1 min), 72 ºC (3 min), followed by a final extension Step at 72 ºC (7 min). The PCR products were separated on 1% w/v

agarose gels, visuvalized under UV illumination and photographed with a digital camera (Gel Doc EQ System, Biorad). Amplicons were later sequenced and compared on the National Center for Biotechnology Information (NCBI) database. The gene sequences of KADR2 then were submitted to NCBI and assigned accession numbers.

Antimicrobial activity

Agar well diffusion method was used to detect the antimicrobial activity of seven isolated strains, against target fish pathogens such as *Aeromonas hydrophila* (ATCC 49140), *Providencia rettgeri* (JX136696), *Aeromonas* sp (JX136697), *Aeromonas* sp (JX136698), and *Aeromonas enteropelogenes* (JX136699). Briefly, 0.1ml of different pathogens was spreader on Mueller Hinton agar plates in which wells with a diameter of 6mm were made and filled with 10^6 – 10^7 CFU ml^{-1} of live suspension of probiotic culture. Plates were incubated at 37 ºC for 24 h and the zone of inhibition was recorded. The strain shown potent antagonistic activity against test pathogens were assessed for their probiotic properties further.

Acid and bile tolerance

The isolated strain were grown overnight at 37 ºC and centrifuged at 8,000rpm for 5 min. The cells were washed twice with sterile Phosphate buffer saline (pH 7.3) and re-suspended in 1ml PBS. Strain were diluted (1:100) in PBS at pH 1, 2, 3 and 4 followed by incubation at 37 ºC and the viability of the bacterial cells were determined in terms of CFU ml^{-1} in *Bacillus* agar plates at different time interval 0, 60, 120 and 180 min. The survivability of the isolates in different pH after 3 h of incubation has also been represented in percentage.

Bile salt resistance of the isolated strain KADR2 were determined by inoculating the growth medium containing 2.5 %, 5.0 %, 7.5 % and 10 % of bile salt followed by incubation at 37 ºC for 3 and 6 h. The growth medium with 0 % bile salt served as control. The treated cells were then evaluated by recording absorbance at 595 nm using ELISA reader (BioRad). Survivability of the isolates was represented by percentage.

Hydrophobicity

Hydrophobicity assay was conducted to evaluate the ability of the strain to adhere the solvent (Thapa et al. 2004). Cells were collected by centrifugation (6000g) from overnight culture and washed with PBS, resuspented in 10 ml Ringer's solution and A_{600} was measured as A_0. Cell suspension was mixed with equal volume of solvent and two phases were mixed by gentle vortexing for 2 min. The aqueous phase was removed after 30 min of incubation at room temperature A_{600} of non aqueous was measured as A_1. The Hydrophobicity of bacterial adhesion to solvent was calculated as $(1-A_1/A_0)$ x100.

Gastric juice tolerance

Gastric juice tolerance was estimated following the protocol described by Ahire et al. (2011). Overnight grown culture (OD_{595} - 0.5) was pelleted by centrifugation and washed twice and suspended in PBS (pH 7.3). The cell suspension was diluted 1:10 in synthetic gastric juice (pH 2.5) and incubated at 37ºC. The survival rate of the isolates were measured after 0, 0.5 and 3 h by spreading on *Bacillus* agar plates, which were then incubated at 37ºC for 24 h and their growth rate was expressed in colony forming units (CFU ml^{-1}) and represented in percentage

Antibiotic susceptibility

The agar overlay method, as described by Cebeci and Gurakan 2003. Cells were grown over night at 37 ºC to obtained OD 0.5. Previously prepared 15 ml of *Bacillus* agar plates and overlaid with 4 ml of soft agar (1%), containing 200 µl of culture. After 1h maintains the plates at room temperature and Antibiotic discs were dispensed on to the plates and incubated at 37 ºC for 24 h. The zone of Inhibition was measured (mm).

Statistical analysis

All experiments were performed in triplicates and results were expressed as mean ± standard deviation

III. RESULTS AND DISCUSSION

Isolation and characterization of probiotics from various resources is a challenging task. Nevertheless recently deciphered role of probiotics in overcoming various infectious diseases in both human and veterinary medicine is prompting the researchers to explore newer probiotic species with diverse potent characteristics. Probiotics are commonly used as alternative medicine for chemical antibiotics and supplemented along with feed in many livestock production sectors. Seven bacterial strains were recovered from *Labeo rohita* gut region of healthy fish showed KADR2 strain possessing inhibitory effect against target fish pathogen (Table 1).

Table I: Antibacterial activities of the isolate from *Labeo rohita* against reference fish pathogens

Name of the Fish pathogens	Test probiotic organisms
	KADR2 (JQ312662)
Aeromonas hydrophila (ATCC 49140)	++
Providencia rettgeri KADR11JX136696	+
Aeromonas sp. KADR12 JX136697	+++
Aeromonas sp.KADR13 JX136698	+
A. enteropelogenes KADR14 JX136699	++

Symbols: + - Zone of inhibition between 1 – 2 mm; ++ - Zone of inhibition between 2 – 4 mm; +++ - Zone of inhibition above 4 mm.

The morphological and biochemical data assures that the isolated strain belong to *Bacillus* sp. by comparing characteristic features as given in the seventh edition of Bergey's manual of deterministic bacteriology. The strain KADR2 are found to be spore forming, oval shaped and terminal under microscope while staining with malachite green. The partial 16S rRNA sequences obtained from the amplification products confirmed that *Bacillus* species. The obtained sequence was submitted to Genbank database and the following deposition number [KADR2 (JQ312662)]. Phylogenitic tree showed close similarity to 16S rRNA gene sequences of *Bacillus infantis* (99%) (Fig.1). 16S rRNA sequencing is the most useful molecular tool to interpret phylogenetic relationships as they are present in the organisms (Woo et al. 2008).

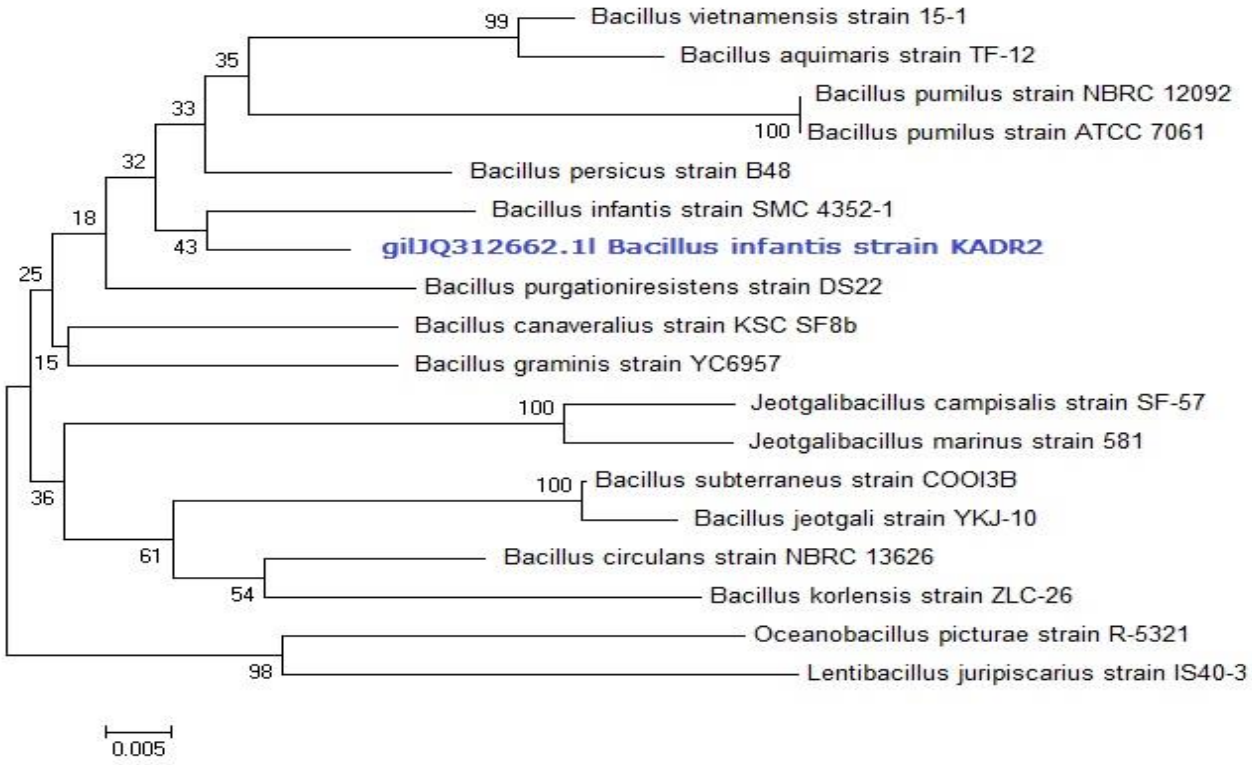

Figure 1: Phylogenetic tree showing species relatedness of *Bacillus* isolates

The tested strain was able to grown at increasing concentration of bile salt (Fig. 2). KADR2 showed survivability of 63.33%, 41.42 %, 38.09 % and 35.71 % at 2.5 %, 5.0 %, 7.5 % and 10 % of bile salt, respectively after 3 h of incubation. However, after 6 h the survivability was reduced to 26.76 %, 21.83 %, 20.19 % and 18.55 %, respectively at increasing concentration of bile salt. An essential trait focusing the beneficial function of strain is their survival in the acidic condition and tolerance to bile salts [FAO/WHO]. Strain KADR2 are able to survive at the lower pH value of 2.0 and the bile concentration 2.5% indicating that the strain can adapt to the conditions of fish GIT at low pH and bile secretion. These findings assure that the isolated strains are able to withstand in the acidic environment and to tolerate the presence of bile salts. In addition to these, antagonistic property is the essential one to qualify the strains as probiotic.

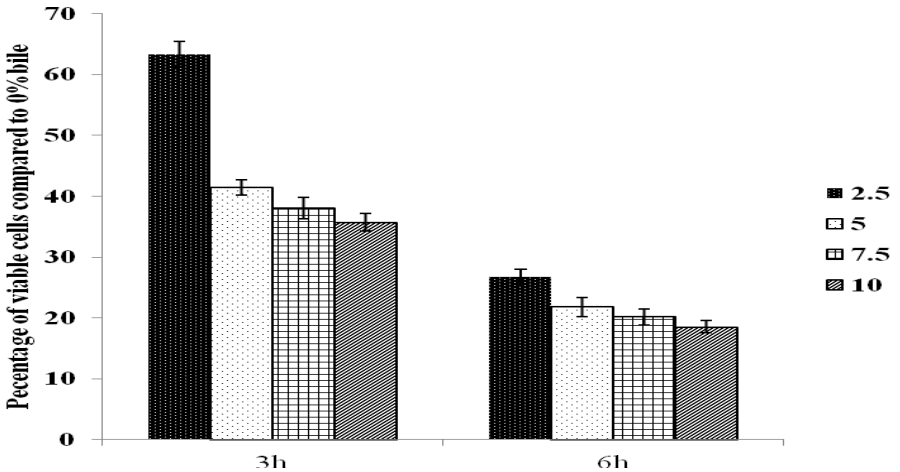

Figure 2: Probiotics isolate bile salt tolerance after 3 and 6h at 37°C. Values are presented as mean ± standard deviation and in terms of percentage

Tolerance of probiotic isolate to gastric juice was evaluated at different time period such as 0.5 and 3 h and was compared to control at 0 min. The viability of the isolate in the presence of gastric juices at different time period is shown in Table 3. KADR2 showed tolerance of 30.76 % after 3 h of incubation when compared to control at 0 min. The hydrophobic cell surface properties of tested strain were examined in xylene, chloroform and ethyl acetate (Fig. 3). The percentages of hydrophobicity were: Xylene at 8.88%; chloroform; 24.14%; ethyl acetate; 21.18%. Adhesion to epithelial cells is another vital parameter to be a potent probiotic, since it provides the ability to resist the flux of the intestinal content (Guo et al. 2010; Tsai et al. 2008). Colonization in intestinal epithelial cell wall and mucosal surfaces is an important desirable property of probiotic bacteria in order to use its beneficial effects. The surface properties like hydrophobicity ability exhibited by isolates may contribute on its adhesion property (Kos et al. 2003). This property could confer a competition to pathogen and colonization of the isolate in the gastro intestinal tract.

Table 3: Gastric juice tolerance analysis of *Bacillus* isolate in terms of CFU ml^{-1}

Name of the isolates	Viability of bacteria in CFU ml^{-1} (×10^3)			% of survivability after 3 h
	0 h	0.5 h	3 h	
KADR2 (JQ312662)	1.04±0.051	0.52±0.060	0.32±0.020	30.76

Each values is the mean ± standard deviation of three separate experiments

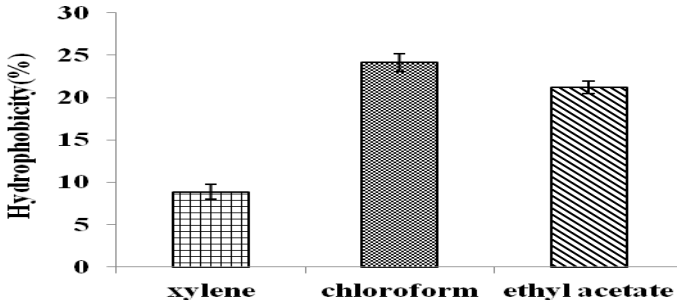

Figure 3: Probiotics isolates cell surface hydrophobicity against various solvents. Each value is the mean ± standard deviation of three separate experiments

The selected isolate was highly susceptible (more than 10 mm of Zone of inhibition) to ampicilllin (10µg), erythromycin (10µg) and 5-9 mm of zone of inhibition to amoxicillin (10µg), cephalaxin (30µg), streptomycin (10µg), penicillin (10µg), gentamycin(10µg), Kanomycin(10µg) and rifampicin(5µg), moderate to chloramphinical(30µg) and tetracycline(30µg) (Table 4). These isolate are susceptible to most of the clinically relevant antibiotics including Amoxicillin, Ampicillin, Cephalexin, penicillin-G and streptomycin etc reveals that, these probiotics microbes are safe to use against fish pathogens in aquaculture industry.

These strains were found to be Catalase positive and hemolytic reactions observe to be non- hemolytic on human blood agar plate after incubation at 37^{o}C for 24 h. The enzyme, catalase is well-known to play a crucial role which reduces the harmful effects of free radicals generated during metabolic process. The probiotic strains in the gut could act as a good antioxidant. (Nishikawa et al. 2009). Absence of haemolytic activity and antibiotic resistance considered one of the good probiotic.

Based on above good attributes, the present study concludes that the identified and characterized the isolate KADR2 are novel, possess notable probiotics properties and thus could be safe to host organism to use as probiotics to enhance livestock production.

Table 4: Antimicrobial susceptibility of the probiotic isolate against the selective antibiotics

Antibiotics (mcg)	**Probiotic isolate**
	KADR2
Ampicillin (10)	+++
Amoxicillin (10)	++
Cephalaxin (30)	++
Streptomycin (10)	++
Penicillin-G (10)	++
Gentamycin (10)	++
Erythromycin (15)	+++
Chloramphenical (30)	+
Kanamycin (10)	++
Tetracycline (30)	+
Rifampicin (5)	++

Symbols: ++ - Zone of inhibition between 2 – 4 mm; +++ - Zone of inhibition above 4 mm

IV. CONCLUSION

Aquaculture needs organisms possessing good acid stability, bile salt, gastric juice tolerance, aggregation property which serves as potential probiotic enhancing immunomodulation and inhibiting the growth of other harmful microorganisms. In this study found that isolate KADR2 presented had good essential probiotics properties, having resistance to acid, bile salt, gastric juice condition, as well as a good capacity for adherence to hydrocarbon, to pathogens, and highly antagonistic effect against fish pathogens which survive in gastrointestinal tract. Therefore this strain can act as potential probiotics enhancing the immunity and inhibiting the growth of other harmful microorganisms. Since *Bacillus* spp., isolated from gastro intestinal tracts of fish should withstand low pH, gastric juice bile, lysozyme, and these characteristics may serve as suitable criteria for probiotic culture selection. In this way KADR2 showed the pre requisite characteristics of probiotic. Hence we conclude that these organisms can be used as probiotics either alone or in combination.

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