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Research Article

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Isolation and characterization of probiotic bacteria isolated from diverse fish fauna of the trodden Vaigai river at Theni district

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ABSTRACT

To determine the probiotic potential of bacterial isolated from fish gut, which enhance the immune system of the host animal and improve the microbial balance of the digestive tract. Fish samples were collected from the trodden vaigai river in Theni district. Seventeen bacterial cultures were isolated from fish gut .Among these, only six bacterial cultures are considered as a probiotic depending on the biochemical and molecular characterization technique. The probiotic characterization tests for acid tolerance, bile salt tolerance, antimicrobial activity and antibiotic test profiles were also determined. The probiotic endosymbiotic bacterial spp prevailing in selected freshwater fishes, namely Oreochromis mossambicus and Labeo rohita .were isolated and characterized by using the 16s rRNA sequencing method. Among the 17 bacterial strains isolated from fish gut samples, 6 bacterial strains were found to be probionts which was confirmed by various probiotic analysis tests. These probionts showed both antibacterial activity and antibiotic susceptibility. Two probiotic bacterial spp such as B. cereus (KU167636) and B. subtilis (KU167639) were selected, based on their beneficiary activities. The overall completed study revealed that the isolated Bacillus spp. fulfills the required criteria for probiotic such as tolerance to harsh conditions such as low pH and high bile salt concentration and it can be produce bacteriocin extra-cellularly which inhibits pathogenic organisms. These isolates were used for potential probiotics.

Keywords: *Bacillus sp* , 16srRNA /Probiota, Antibiotic susceptility, Antimicrobial activity, vaigai river.

INTRODUCTION

The main tributaries of the river Vaigai are, the river Suruliyaru, the river Mullaiyaaru, the river Varaganadi, the river Manjalaru and river Kridhumaal. All these rivers, except Kridhumaal join with the great Vaigai river nearer to the places around the Vaigai dam which is situated in Theni district, Tamil Nadu, India whereas Kridhumaal joins Vaigai in Madurai. Vaigai gets major feed from the Periyar dam in Kumuli, Kerala. The Periyar River water in Kerala is diverted into the Vaigai Riverin Tamil Nadu via a tunnel through the Western Ghats.

The term “probiotic” which literally means “for life” has since been employed to describe these health-promoting bacteria. The World Health Organization has defined probiotic bacteria as “live microorganisms which when administrated in adequate amounts confer a health benefit on the host”. The use of probiotics in the culture of aquatic organisms is increasing with demand for more environment friendly aquaculture practices (Gatesoupe 1999). Aquaculture has made significant advances in recent years in the production of a wide range of aquatic organisms, both for human consumption and as ornamental species (Balcázar et al 2006; Kesarcodi-Watson et al 2008).The most commonly used probiotic is usually characterized as gram positive, non motile, non sporulating bacteria that

produce lactic acid as their main byproduct due to fermentation (Vijayaram et al 2014). The use of antibiotics to Cure bacterial infection and prevent fish mortality in aquaculture is becoming limited as pathogens develop resistance to the drugs (Gonzalez et al 2000; Gomez-Gil et al 2000).

Probiotics such as *Lactobacillus* spp. are reported to have inhibitory activity against common human pathogens (Murry et al 2004; Raja et al 2009; Moghaddam et al 2006). They are able to produce antimicrobial substances such as bacteriocins, which have great potential to be used in therapeutics and as food bio-preservatives (Mobarez et al 2008).

In the present study is focusing to isolate and characterize the indigenous of *Bacillus* sp from trodden vaigai river fish gut and to asses the antibacterial activity against human and fish pathogens in vitro.

EXPERIMENTAL SECTION

Isolation of bacteria from fish gut

Live fish samples were collected from trodden vaigai river, tamil nadu, India. The collected samples were transported in sterilized polythene bags containing habitat water. The fish samples were identified using standard reference manuals (Zacharias et al 2013). Among the selected fish *Oreochromismossambicus* (Tilapia) and *Labeo rohita* (rogu) Fishes were washed with sterile distilled water to remove any undesired dusty materials. The gastrointestinal (GI) tract of the fishes was dissected under sterilized conditions. Further, the GI tract was homogenized using sterile distilled water and centrifuged at 13,000 rpm for 10 minutes. After centrifugation, the supernatant was taken and serially diluted with sterile distilled water. From the serially diluted samples, 10^{-7} samples were selected and inoculated on Nutrient Agar plates and incubated at room temperature for 24 h. The microbial colonies were separated using the quadrant streaking method. Glycerol stocks of individual isolates were maintained in deep freezer for further use (Ghosh et al 2007).

Biochemical characterization

The isolated microorganisms were characterized by physiological and biochemical tests such as Gram reaction, catalase test[Norris et al 1981], oxidase test, simmons citrate test, Indole test, amylase test (Arvinder et al 2012) and carbohydrate fermentation test were performed [Difco Manual 1984] according to the criteria of Bergey's Manual of Systemic Bacteriology (Boone et al 2005).

DNA isolation and 16S rRNA sequencing

Overnight culture of bacterial cells was isolated from fish and it was centrifuged at 12,000 rpm for five minutes. The supernatant was discarded and the pellets were air dried. The pellets were re-suspended in 100µL of TE buffer (pH 8) and 120µL of lysosyme (10 mg/mL) and it was incubated for one hour at 37ºC for cell lysis. Genomic DNA was isolated by the HIPURA Genomic DNA purification Kit. Later, bacterial 16S rDNA was amplified from the extracted genomic DNA by using the universal bacterial 16S rDNA primers, forward primer- (5' AGAGTTTGATCCTGGCTCAG-3') and reversere primer-GGTTACCTTGTTACGACTT(5' -3'). PCR was performed with a 50-µl reaction mixture containing 1 µl (10 ng) of template, 0.5µg of each primer, 1.5 mM $MgCl_2$, and 50mM dNTP (deoxynucleoside triphosphate), 1U of Taq-polymerase and buffers as recommended by the manufacturer (Fermentas, Hanover, Germany) with the cycling parameters typically being with 94°C for 60 sec, 55°C for 1min, and 72°C for 2 min (35 cycles) using Cyber-Lab® PCR system. PCR products were analyzed by electrophoresis in 1.5% (w/v) agarose gel in 1x TAE buffer with ethidium bromide (0.5 mg/ml).

Nucleotide sequence accession numbers and phylogenetic analysis

Partial sequences of 16S rRNA genes of selected bacterial isolates (1-8) were submitted to GenBank and have the following accession numbers: KU167635, KU167636, KU167637, KU167638, KU167639, and KU167640 . Sequences were matched with previously published bacterial 16S rDNA sequences in the NCBI databases using advanced BLAST (Altschul et al 1994). Based on the scoring index the most similar sequences were aligned with the sequences of other representative bacterial 16S rDNA regions by using ClustalX software version 1.83 (Thompson et al 1994; Jeanmougin et al 1998). Further phylogenetic analysis was performed by using Mega 5.0 software.

Screening for probiotic properties

Acid and bile salt tolerance

A probiotic microorganism must overcome physical and chemical barriers in the gastrointestinal tract of fishes. Therefore, in this study acid tolerance property of the isolate was determined by following the procedure described by (Erkkila et al 2000). The isolate was grown in nutrient broth for 24 hours at 30˚C. After incubation, cells were

harvested by centrifugation were at 10,000 rpm for 10 min, then it was washed and re-suspended in 1 ml of sterile phosphate buffered saline (PBS) at different pH for various time intervals (0, 60, 120, 180 min). Later the mixture was transferred to fresh nutrient broth and incubated at 30˚C for 24hrs. The growth of bacteria was measured at 560nm and the survival percentage of strain to different pH was calculated. The bile salt tolerance of the isolate was determined as per [$_{21}$]Nutrient broth (100 ml) supplemented with different concentration of bile salt (wt/vol. ox gall) was prepared and inoculated with one ml (3×10^7 cells ml^{-1}) of the isolate and incubated at 30˚C. After incubation the growth of bacteria was measured (A_{560}nm) at different time intervals and the survival percentage of the isolate was calculated.

Auto-aggregation and solvent adhesion assay

Auto-aggregation and adhesion of bacterial cells to different solvents was calculated as per the procedure described by (Menghe Bilige et al 2009; Del Re et al 2000) respectively. The isolate was grown in nutrient broth for 24 hours at 30 °C. The cells were pelleted, washed twice with PBS (pH 7.3), resuspended in the same buffer to get an absorbance (A_0) value of 0.5 at 600 nm. For auto-aggregation assay, the bacterial suspension was incubated for different time durations and absorbance was measured (A_1). For the solvent adhesion assay, 3 ml of cell suspension was mixed with 1 ml of petrol and incubated for 20 min. After incubation, the absorption was measured as A_1.The percentage of auto-aggregation and adhesion to different solvents was calculated as $(A_0 - A_1/ A_0) \times 100$.

Antibiotic susceptibility and haemolysis test

The susceptibility of isolate to different antibiotics was determined by placing standard antibiotic discs (Hi Media, Mumbai) on the surface of Muller Hinton agar medium seeded with a lawn of the isolate. Plates were observed for the zone of inhibition after 24 hrs incubation at 30ºC. The hemolytic ability of the isolate was examined on nutrient agar plate supplemented with 5% Sheep blood after 24 hrs incubation at 30°C.

Antimicrobial test

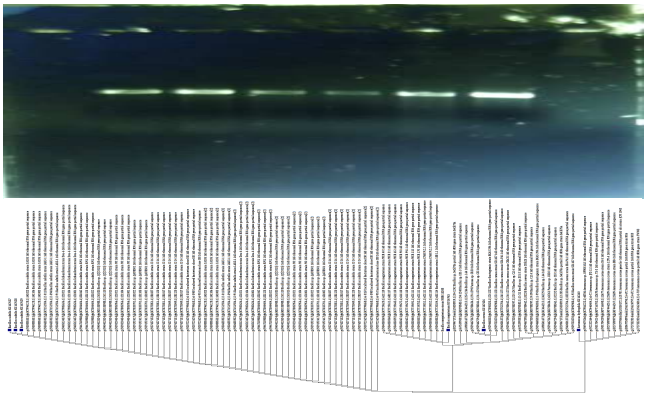
The antagonistic property of Bacillus sp was performed according to (Ahire et al 2011) method briefly the pathogenic strains were swabbed in to a nutrient agar medium and discs which were coated with bacillus strain was placed over. The plates were incubated at 37°C and examined for clearance around the disc. A clear zone around the disc suggest for the antimicrobial activity of the isolate.

RESULTS AND DISCUSSION

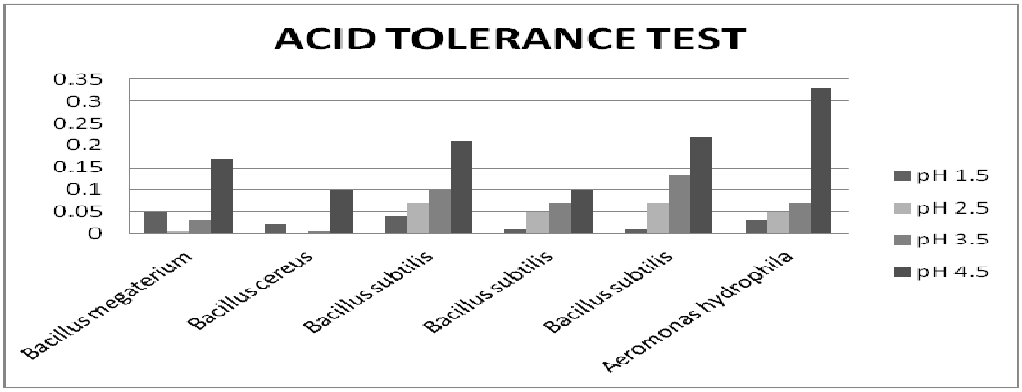
The gut of the fish species, namely *Oreochromis mossambicus* and *Labeo rohita*from Trodden vaigai river, theni district were surgically removed under aseptic condition. Seventeen bacterial strains were isolated from fish gut. In the present studies revealed that all the isolated strains were gram positive and rod shaped.

Among them six bacterial strains were considered as probiotic based on their hemolytic, acid and bile tolerance properties. The goal of this research work was to isolate and characterize potential probiotic bacteria from yoghurt samples of Bangladesh and to assess their anti-bacterial activity against some common pathogenic bacteria. Based on the morphological characteristics four (4) isolates were identified as *Lactobacillus* spp. from yoghurt samples. After gram staining the isolated bacteria were rod shaped, convex, rough, smooth, shiny, irregular, circular, gram positive, facultative anaerobic, non-spore forming which indicate them to be the member of *Lactobacillus* spp (Bauer et al 1966). Survival in the extremely low pH is one of the major selection criteria for probiotic strains. Although in the stomach, the pH level was low as 2.5, in most *in vitro* assays pH 4.5 has been preferred. For selection the strains resistant to low pH, used medium buffered with PBS to corresponding pH.

(Fig-1)

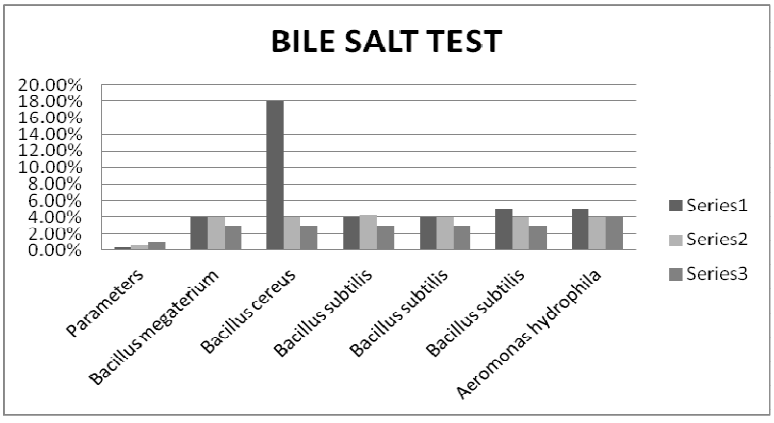


(Fig-2&3)



(Fig-4)

The probiotic bacterial strains were cultured on medium with varying pH (say pH 2.5, 3.5, 4.5 and 5.5) for three hours (median log phase) to study the acid tolerance effect on their growth of these bacterial strains. After examinations, the bacterial strains that were able to survive at pH 2.5 was selected for further study. Based on our observation *B.cereus* (KR067665) *and B. subtilis* (KR708822) showed better tolerant as compared to other bacterial strains. Response to a one-unit pH shift in experimental batch culture inoculated with fish gut microbiota is considered significant. In the present study revealed that the isolation of probiotic bacteria from fish gut samples of trodden vaigai river and to asses their antibacterial activity against human and fish pathogenic bacteria. According to the morphological characterization of 6 isolates were identified 5 species are *Bacillus* sp and one sp are aeromonas. The significant growth of the isolates at pH 6.5 on MRS – agar plates in anaerobic conditions further confirmed their identification as *Lactobacillus* spp. (Holt et al 1994).

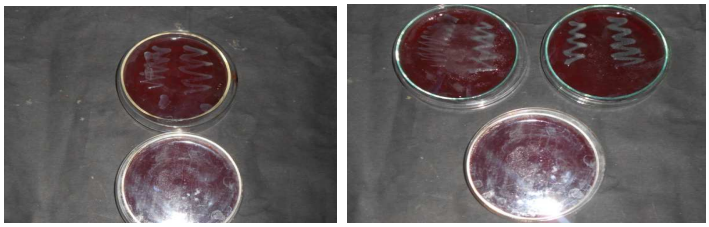


(Fig-5)

The bacterial strains resistant to low pH were screened for their ability to tolerate against the bile salt. The bile concentration of the human gastrointestinal tract varies, the mean intestinal bile concentration is 0.3% w/v. Bacterial strains that are able to tolerate bile salt was checked by growing them in different concentration of bile salts (0.3%, 0.5% and 1.0%) for the growth period and observed by using UV-Visible spectrophotometer at 600nm.

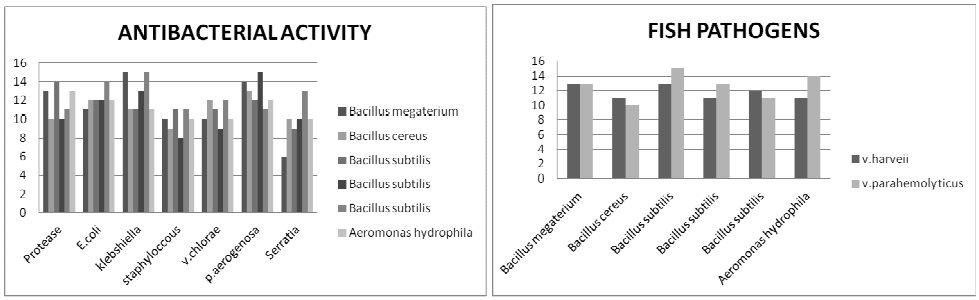
The results of the present study reveals that the growth of the isolates was more in 0.3% of bile salt whereas growth at 0.5% and 1.0% bile salts were less. According to the results, the isolates *B. cereus* (KR067665) *and B. subtilis* (KR708822) were resistant to 0.3% bile salt that poses them as an effective probiotic strain. The isolated *Lactobacillus* spp. can tolerate a wide range of pH (1-9) and grow well at acidic pH (1-5). The 0.3-1% bile salt were supplemented in the growth media, as it corresponded to that found in the fish intestinal tract and 0.3% is the maximum concentration that is present in fish gut (Elizete et al 2005). Therefore, before selection of probiotic bacteria for human consumption it must be endurable to 0.3% bile concentration (Graciela et al 2001). *Lactobacillus* spp. isolated in this study was resistant to 0.3% bile salt. All of the isolates are able to survive and grow in 0.3% bile salt concentration.in this present study the bile salt concentration 0.3-1% the low concentration bile salt the bacterial growth rate is high.the high salt concentration bacterial growth rate is decrease.

The microscopic results were observed that all the isolated probiotic bacterial strains were Gram positive and rod shaped. The majority of the probiotic bacteria are from Bacillus sp. (either *Bacillus cereus* or *Bacillus subtilis*). However, isolated *Pseudomonas flouresence* from *Punitusmelanampyx* (kudukonda). Owing to high resemblance on species diversity, two effective isolates were selected (named as *B.cereus* (KR067665) *and B. subtilis* (KR708822)) for further evaluation based on their acid and bile
tolerance ability.



(Fig-6)

The selected isolates were non-pathogenic based on negative result obtained from haemolytic activity, (i.e.) clear zone was not formed around the bacterial colonies when inoculated in Blood agar medium. Apart from their Non-hemolytic behavior, notable features of selected strains include: (1) Both selected strains were able to use Citrate as its sole carbon source. (2) Catalase activity reveled both the stains are able to breakdown the H_2O_2. (3) *Bacillus cereus* (KR067665) was homo-fermentative whilst *Bacillus subtilis* (KR708822) was hetero-fermentative. (4) *Bacillus cereus* (KR067665) can produce positive acid production using all carbohydrate sources with exception to mannitol, whereas, *Bacillus subtilis* (KR708822) showed positive acid production for all the carbohydrates. (5) *Bacillus cereus* (KR067665) and *Bacillus subtilis*(KR708822) showed positive for Amylase activity and Vogues Prousker test and negative for Indole and Methyl red test. The Oxidase, catalase and IMViC test of selected isolates gave the same results as *Lactobacillus* spp. All of the isolates were Indole, MR, VP, Citrate, Oxidase and Catalase negative, the results are similar to the findings of (Dhanasekaran et al 2010). All the isolates were Indole, methyl red, catalase negative the results are similar. Among the carbohydrates used in this study, all the four isolates were able to ferment glucose, sucrose, fructose, lactose, xylose, Ribose, galactose, maltose, mannitol, trehalose, rhamnose and dextrose. It is evident that they are able to grow in a variety of habitats utilizing different type of carbohydrates. The current study explored the carbohydrate fermentation are used glucose, sucrose, arabinose and maltose. The pH is an important factor which can dramatically affect bacterial growth. To be used as probiotic, organisms were resistant to low pH of human gut. The isolated *Lactobacillus* spp. can tolerate a wide range of pH (1-9) and grow well at acidic pH (1-5).



(Fig-7A&B)

The observed results of the antimicrobial effect of the isolates against selected human and fish pathogen shows that majority isolates were very low inhibitory activity for human pathogens such as *E. coli, Klebshiella spp, Bacillus spp, Proteus mirabilis* and *Staphylococcus aureus*. Among the two lead strains *B. cereus* (KR067665) *and B. subtilis* (KR708822) that are under investigation showed significant inhibitory activity against human pathogen *Serratia marcescens*. In addition, all the probiotic strains are efficient in inhibiting the growth of fish pathogen *Vibrio harveyii*. Inhibition of *V. parahemolyticus* is significantly lower for all the probiotic microorganisms except *P. fluorescences*, which showed a significant inhibitory activity. Antimicrobial activity is best selection criteria for probiotics. Antimicrobial effects of lactic acid bacteria were incurred by producing some substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins (Quwehand et al 2004). Probiotics including *Lactobacillus*, Bifidobacterium and *Streptococcus* spp. were known to be inhibitory to the growth of a wide range of intestinal pathogens in human. In addition to the favorable effects against disease caused by an imbalance of the gut microflora, many experimental observations were showed a potential protective effect of probiotic bacteria against the development of colon tumors (Elizete et al 2005).The results of the antibiotic sensitivity indicate that majority of organisms were sensitive to tetracycline, cephalothin and lincomycin. On contrary, majority of organisms were resistant to Penicillin-G and Amoxicillin at lower concentration (Fig.- 8).

CONCLUSION

The overall completed study revealed that the isolated *Bacillus* spp. fulfills the required criteria for probiotic such as tolerance to harsh conditions such as low pH and high bile salt concentration and it can be produce bacteriocin extra-cellularly which inhibits pathogenic organisms. These isolates were used for potential probiotics.

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