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Probiotic properties of bacterium isolated from shrimp cultured ponds

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***Abstract*-**Use of probiotic bacteria in aquaculture has tremendous scope and the studies of the application of probiotics in aquaculture have a glorious future. The present study was aimed at the studies on the probiotic characters of bacteria which were isolated from shrimp cultured ponds. Initially 26 soil samples were collected from dried shrimp cultured ponds; bacteria were isolated by serial dilution. 10 samples were able to produce prominent colonies which were further subjected for repeated sub culturing for pure cultures on nutrient agar medium. Among 10 isolates six were Gram positive, and four of them were Gram negative. All isolates fermented glucose, three of them (HL1,HL2,HL6) fermented lactose, four (HL6,HL4,HL5andHL6) fermented xylose, six (HL1,HL2,HL4,HL8,HL9,HL10) gave positive result for catalase, four (HL1,HL2,HL4,HL6) showed positive result for urease test and four (HL1,HL2,HL3and HL4) gave positive result for nitrate reductase test. Probiotic bacteria such as lactic acid bacteria are mostly gram positive so only six gram positive strains were made pure culture on MRS agar which were further characterized for their probiotic nature. All the isolates survived under low pH (2and3) for 1hr and 6hrs. All isolated colonies survived under 0.3%, 0.5% and 0.1% oxgall treatments, HL1 (98±1.6cfu/ml) and HL5 (92±4.2cfu/ml) showed high survival rate under 0.3% concentration. All isolates survived in gastro intestinal juices for 4hrs, HL1 showed high survival rate 72±1.2cfu/ml. High cholesterol assimilated by HL5 (27±1.2cfu/ml). DNA was isolated from five strains except HL2 and DNA amplification of V2-V3 variable region for identification of *Lactobacillus* species.

Key words: Probiotic, urease, oxgal and cholesterol

I. INTRODUCTION

Aquaculture has become the world's fastest growing foodproduction sector during last few decades. But according toWorld Bank report global losses from shrimp diseases were around 36 billion USD . Because of negative consequences of using antibiotic in aquaculture such as indiscriminate use has led to the appearance of antibiotic resistant strains (Skjermo and Vadstein,1999) and also the contamination of shrimp meat and environment (Holmstrom et al., 2003). These have led to suggestions that the use of non pathogenic bacteria as probiotics (Vaseeharan and Ramaswamy, 2003). Now adays in aquaculture industry dietary supplementation of probiotic bacteria has been widely employed (Gatesoup, 1999 and Vine et al., 2006).

Probiotics are a live microbial feed supplement that beneficially affects the host animal by improving its intestinal balance (Fuller, 1989). Another definition given by Coeuret et al. (2004) that probiotics are live micro organisms that upon digestion certain numbers exert health benefit beyond inherent nutrition.

Many types of probiotics are economical force in aquaculture, they are gram negative, gram positive, bacterio phages, yeasts and unicellular algae, (Irianto and Austin, 2002). Probiotic bacteria can prevent the damage or disease by pathogenic bacterium in host by producing antimicrobial substances that inhibits the growth or attachment by harmful bacteria by out competing the pathogen for nutrients or by modulating immune response (Moriarty, 1998). The use of terrestrial bacterial species as probiotics for aquaculture have had limited success, because bacterial strain characters are dependent up on environment in which they thrive. So the better approach is isolating potential probiotic bacteria from the marine or pond environment in which they grow. The present research was aimed at isolation of bacteria from dried shrimp cultured ponds and their characterization under various physical and physiological conditions.

II. MATERIAL AND METHODS

Collection of soil samples

Twenty six (26) soil samples were collected from the brakish water shrimp ponds from Mypadu, Ramudu palem and Kudithipalem of Nellore district, Andhra Pradesh, India. All these areas are very nearer (20 km away from Bay of Bengal) where extensive *P. monodon* culture being practiced for the last 20 years and *P. vannamei* culture being practiced for the last five years.

Isolation and enumeration of bacteria

All the samples were transferred to laboratory under sterilized conditions. All soil samples were subjected for serial dilution under extreme sterile conditions using nutrient agar supplemented with 15% sodium chloride. All the prominent bacterial colonies obtained were subjected for pure culture isolation. An inoculum (0.1 ml) of each decimal dilution of samples was plated onto the surface of De Man, Rogosa and Sharpe (MRS) agar (Difco, Detroit, MI, USA) which were incubated anaerobically in anaerobic jar (BBL, Gas Pak Plus).

The samples were cultivated under sterile condition in MRS agar plates and they were incubated at 37^0Cfor 48hrs. Each isolates were cultured in MRS agar again and preserved in refrigerator in order to conserve them.

Identification of the bacterial isolates

Representatives of bacterial colony types from MRS agar were isolated and identified using conventional morphological and biochemical tests according to Bergey's manual of determinative bacteriology (Holt et al., 1994).

Biochemical characterization

The bacteria isolated were biochemically characterized by grams staining, carbohydrate fermentation, catalase production, nitrate reduction and urea production.

Probiotic properties of Isolates

For determination of probiotic properties all the isolates were subjected for the analysis to look for the probiotic characters such as resistance to low pH, tolerance against bile salt tolerance to gastro intestinal juices and cholesterol assimilation

Acid tolerance

Acid tolerance was determined according to the method described by Hyrominus et al. (2000). Cells of *Lactobacillus sps* strains were grown in MRS broth at 37°C overnight, and sub cultured in fresh MRS (3%, v/v) broth adjusted to pH 2.0 and 3.0 with hydrochloric acid (3.0 mol/L). Survival rate was calculated as the percentage of the cfu/ml after incubation at 37°C for 60 and 180 min compared to the cfu/ml at time 0 min.

Bile tolerance (Oxgall treatment)

Bile tolerance test was conducted using a modified method of Gilliland et al. (1984). Overnight cultures of *Lactobacillus* strains were inoculated (3%, v/v) into MRS broth and MRS broth containing 0.3, 0.5 and 1.0% (w/v) oxgall. Survival rate was calculated as the percentage of the cfu/ml after incubation at 37°C for 240 min compared to the cfu/ml at time 0 min.

Tolerance to simulated human gastrointestinal tract

In vitro determination of viability under conditions similar to those prevailing in the GIT was performed according to the method of Charteris et al. (1997). Briefly, simulated gastric and pancreatic juices were prepared by suspending pepsin (3 mg/ml; Sigma) and pancreatin USP (1 mg/ml; Sigma) in sterile sodium chloride solution (0.5%, w/v), and adjusting the pH to 3.0 and 8.0 with hydrochloric acid (3.0 mol/L) and NaOH (1 mol/L) , respectively. Portions (0.2 ml) of washed cell suspensions of the isolated bacterial strains in phosphate buffered saline (PBS, pH 7.0) were inoculated in 1.0 ml of simulated gastric or pancreatic juice and 0.3 ml NaCl (0.5%, w/v), mixed and incubated at 37°C. Total viable counts (cfu/ml) were evaluated after incubation for 180 min in cultures tested for gastric transit tolerance, and for 240 min in cultures tested for small intestinal transit tolerance.

Assay of cholesterol assimilation

Freshly prepared sterile MRS broth was supplemented with 0.3% (w/v) oxgall and 0.2% (w/v) sodium thioglycolate. Water–soluble cholesterol (polyoxyethanyl-cholesterylsebacate, Sigma) (Pereira and Gibson, 2002a) was filter sterilized and added to the broth at a final concentration of 100 μg/ml. Bacterial strains isolated from probiotic samples were inoculated (3%, v/v) into 5 ml of the Ch-MRS-Thio broth, and incubated anaerobically at 37°C for 20 h. Uninoculated sterile broth was used as the control. After incubation, cells were removed by centrifugation (10000 g, 4°C, and 10 min) and the remaining cholesterol concentration in the broth was determined using a modified σ-phthaladehyde method of Ruddel and Morris (1973).

Isolation of lactic acid bacteria from selected probiotic samples and DNA extraction

Out of the 6 samples five samples were subjected for molecular characterization leaving HL2, as it was a poor performer in tolerance tests performed for probiotic characterization. The 5 selected probiotic samples (HL1, HL3, HL4, HL5, HL6) selectedwere made in sterile physiological saline, plated onto MRSagar (Biolab Diagnostics, Midrand, South Africa) supplemented with natamycin, and incubated at 30 °C for 24–48 h. Colonies were harvested from plates representing 10^3 CFU/ml and suspended in10 ml of sterile physiological saline. DNA was isolated from 2 ml of cell suspension Dellaglio et al., 1973.

DNA amplification for lactobacillus identification

The V2–V3 variable region (approx. 200 base pairs) of the 16S rRNA gene in lactic acid bacteria was amplified by using primers 534 (5' ATTACCGCGGCTGCTGG 3') and 341FGC (5'GCAGGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC ACGGGGGGCCTACGGGAGGCA 3'). The PCR reaction was performed in 50 μL of PCR mixture containing 0.5 mM of primers, 200 mM dNTP (Takara Bio Inc., Shiga, Japan), 0.5 U Taq DNA polymerase (Takara Bio Inc., Shiga, Japan), 1´PCR buffer (TakaraBio Inc., Shiga, Japan) and 10 μL of DNA. The following conditions were used: initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and elongation at 72 °C for 8 min. PCR reactions were performed in a Master Cycler TM Thermal Cycler (Eppendroff, Germany). Amplicons were analyzed on 2% (by mass per volume) agarose gels with ethidium bromide and 0.5TB buffer. DNA fragments were visualized under UV light. The two positive samples for 16s rRNA gene were used in the entire study for the health management and water quality maintenance during the hatchery practices and in farming.

III. RESULTS

Among the 26 samples serially diluted only 10 samples were able to produce prominent colonies which were further subjected for repeated sub culturing for the isolation of pure colonies. A total of 10 pure colonies obtained further streaked on MRS agar slants for further studies.

Biochemical characterization

All isolates were subjected to gram staining the gram reaction of isolates were observed under light microscope HL1, HL2, HL3, HL4, HL5 and HL6 were shown to be gram positive and the remaining isolates were gram negative. All isolates produced gas

from glucose. HL1, HL2, HL 6 fermented the lactose. HL1, HL 4, HL5, HL6 fermented the xylose. HL1, HL2, HL4, HL8, HL9, HL10 gave positive result for catalase test.HL1, HL2, HL4, HL6 showed positive result in urease test and in nitrate reductase test HL1, HL2, HL3, HL4 gave positive result. The positive and negative results for these biochemical characterizations were given in the **Table-1**

Acid tolerance test
All the isolates were subjected for acid tolerance test under different incubation periods. The results (Table-2) have shown that all the isolates tolerated low pH of 2 and 3 for 1hr and 6hrs. All the isolates were resistant to low pH, the survival rates of isolates at pH2 and pH 3 for 1hr and six hrs were given in. HL 2 showed highest survival rate at both pH2 and pH3 that is 91±1.2cfu/ml and 90±2.2cfu/ml, HL 4 showed least survival rate 64±3.7cfu/ml and 54±3.5cfu/ml.

Tolerance against Bile
The strains were further screened for their ability to tolerate the bile salt. Under 0.3% concentration HL1 and HL5 showed highest survival rate 98±1.6cfu/ml and 92±4.2cfu/ml respectively, and at 0.5% concentration also HL1 and HL5 showed highest survival rate 94±1.7cfu/ml and 90±3.2cfu/ml respectively. Under 0.1% concentration HL1, HL4 and HL5 showed highest survival rate 93±1.4cfu/ml, 87±2.2cfu/ml and 84+/-1cfu/ml . All the isolates were able to grow in 0.3%, 0.5% and 0.1% for 4hrs (240 min) (Table-3).

Survival rate in gastric and intestinal juices
All isolates were survived in gastric and intestinal juices at both pH3 and pH8 for 4hrs. And all isolates were efficiently survived at pH 8 than pH3. The results were shown in Table-4. Among the six isolates at pH 3, HL 1 has high survival rate 72 ± 4.2cfu/ml and the least 52 ± 2.1cfu/ml shown by HL 6. At pH 8 HL 1 has highest survival rate 92 ± 2.7cfu/ml followed by HL 3 that is 91 ± 1.9 cfu/ml and the least was recorded by HL 5 that is 69 ± 3.7cfu/ml.

Cholesterol assimilation
All six isolated strains were tested for cholesterol assimilation. These results were showed in Table-5. The highest assimilation activity 27.0±1.2µg/ml was recorded in HL 5, followed by HL 3 (22±1.7µg/ml), and HL 1 (20 ±1.6µg/ml). The least 13 ± 1µg/ml was recorded in HL 2.

Molecular identification of lactobacillus species
After biochemical characterization, *Lactobacillus* bacteria were subjected for the molecular identification of the 16s rRNA gene using the specified primers and conditions mentioned in the materials and methods section. Bacterial DNA samples isolated were found to have the V2-V3 sequence of *lactobacillus* which is of approximately 200 bp fragment (Fig.1). All the screened samples were shown to be amplified for V2-V3 region.

IV. Discussion

Resistance to low pH is one of the major selection criteria for probiotic strains (Cakir, 2003), because in the stomach the high concentration of bile components in small intestine of host can influence the probiotic strains selection (Hyronimus et al., 2000). In the present study all the six isolates were resistant to low pH for 1hr and 6hrs time duration. Jatindra et al., 2010 selected 55 acid tolerant strains of LAB in PBS buffer P^H-2.5 for 3hrs. Succi et al. (2005) also reported the *Lactobacillus rhamnous* strain survival at pH-3.0 after 2hrs period. The results of present study also are in agreement with the above reports.

Resistance against bile salt and survival in gastric juices at pH 3 and pH 8 is next important criteria for colonization and metabolic activity (Strompfova and Laukova, 2007). The mean bile salt concentration of human or animal gastrointestinal tract is about 0.3% so it is considered as high enough and critical to screen for resistant bacterial strain (G.R.Goldin et al., 1992). According to Maragkoudakis et al. (2005) all isolated strains tolerated 0.3% bile salts concentration in 4hrs. The results of present study showed that all six isolates were resistant to low pH also showed high survival rate at 0.3% concentration as well as at 0.5% and 0.1% bile concentration for 4hrs. Among all isolates HL1 and HL5 showed high survival rate at all concentration. These two strains also showed high survival rate at pH 8 in the simulated gastric and intestinal juice. *Lactobacillus* strain MG2-1 showed highest tolerance at pH 8 for longer time among all isolates to the artificial gastrointestinal juice and bile salts (M.Bilige et al., 2009).

A good probiotic bacterium should have cholesterol reduction efficiency. M.Bilige et al. (2009) isolated 30 *lactobacillus* strains, MG2-1 have high cholesterol removal rate (51.74±0.04%). According to Nagpal et al. (2012) probiotic have many health biological properties, one of them was anti cholesterol assimilation because elevated levels of certain blood lipids are a greater risk for cardiovascular disease. Lavanya et al. (2011) found two isolates16, 43 and *L.brevis* has ability to reduce the cholesterol level up to 80% in 24hrs. Another report showed lactic acid bacteria can reduce the serum cholesterol level up to 50% in the presence of bile salt in 48 hrs (Gulsandi et al., 2003). In the present study the highest assimilatory activity was 23±1.2µg/ml which was recorded in HL 5. This is because ability of organism to reduce cholesterol level was due to assimilation of cholesterol with in bacterial cell and increased excretion of bile salts due to deconjuction by the bile salt hydrolase (Salminen et al., 2002).

All the selected samples for amplification were able to produce amplificons of 200 base pairs except HL6 which was failed in V2-V3 region amplification. Similar results were obtained by *L. plantarum L. fermentum, L. sakei by* Svetoslav et al. (2009) during the evaluation of *Enterococcus mundtii* ST4V (a potential probiotic and bacteriocin-producing strain), during its survival in commercial boza.

V.CONCLUSION

From the results obtained it can be concluded that among the 10 samples only six samples HL1, HL2, HL3, HL4, HL5 and HL6 are suitable bacteria in order to be used as probiotic bacteria terms of resistance to physical factors, their potential to grow in bile salts, gastric and intestinal juices and the ability to assimilate cholesterol**.** When tested for V2-V3 variable region of

lactobacillus HL6 failed to amplify the specified 200 base pairs amplicon. Hence from the results it can be concluded that HL5 can be considered as best probiotic as it was found to have high tolerance capacity and high cholesterol assimilation.

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Table 1: Characterization of bacteria

Test	HL1	HL2	HL3	HL4	HL5	HL6	HL7	HL8	HL9	HL10
Grams staining	+	+	+	+	+	+	-	-	-	-
Catalase	+	+	-	+	-	-	-	+	+	+
Urease	+	+	-	+	-	+	-	-	-	-
Nitrate reductase	+	+	+	+	-	-	-	-	-	-
Glucose fermentatio	+	+	+	+	+	+	+	+	+	+
Lactose fermentation	+	+	-	-	-	+	-	-	-	-
Xylose	+	-	-	+	+	+	-	-	-	-

Table2: Survival rate of the *Lactobacillus* species under acidic conditions

Strain	Survival at pH 2 (1hr)	Survival at pH 3 (1hr)	Survival at pH 3 (6hr)
HL 1	77+/-3.2	75+/-3.1	71+/-2.1
HL 2	91+/-1.2	90+/-2.2	88+/-1.7
HL 3	84+/-3.2	81+/-2.1	61+/-4.1
HL 4	64+/-3.7	54+/-3.5	40+/-6.5
HL 5	75+/-3.2	70+/-2.2	55+/-2.2
HL 6	76+/-3.2	72+/-3.0	70+/-2.0

Table3. Survival rate under different concentrations of oxgall treatment (Bile tolerance test)

Isolated Strain	0.3%(240 min)	0.5%(240 min)	1%(240 min)
HL 1	98+/-1.6	94+/-1.7	93+/-1.4
HL 2	86+/-2.7	80+/-2.9	76+/-2.2
HL 3	89+/-4.4	88+/-4.4	81+/-2.7
HL 4	89+/-4.6	87+/-4.2	87+/-2.2
HL 5	92+/-4.2	90+/-3.2	84+/-1.0
HL 6	89+/-3.9	82+/-3.9	80+/-3.9

Table 4. Survival rate in gastric and intestinal juices

Isolated Strain	Survival rate in gastric juice(pH 3)(240 min)	Survival rate in intestinal juice(pH 8)(240 min)
HL 1	72+/-4.2	92+/-2.7
HL 2	53+/-3.1	73+/-2.1
HL 3	71+/-2.1	91+/-1.9
HL 4	66+/-4.2	76+/-4.2
HL 5	64+/-3.2	69+/-3.7
HL 6	52+/-2.1	82+/-4.0

Table 5. Cholesterol assimilation

Isolated Strain	Cholesterol assimilation (µg/ml)
HL 1	20.0+/-1.6
HL 2	13.0+/-1.0
HL 3	22.0+/-1.7
HL 4	20.0+/-1.9

HL 5	27.0+/-1.2
HL 6	16.0+/-2.2

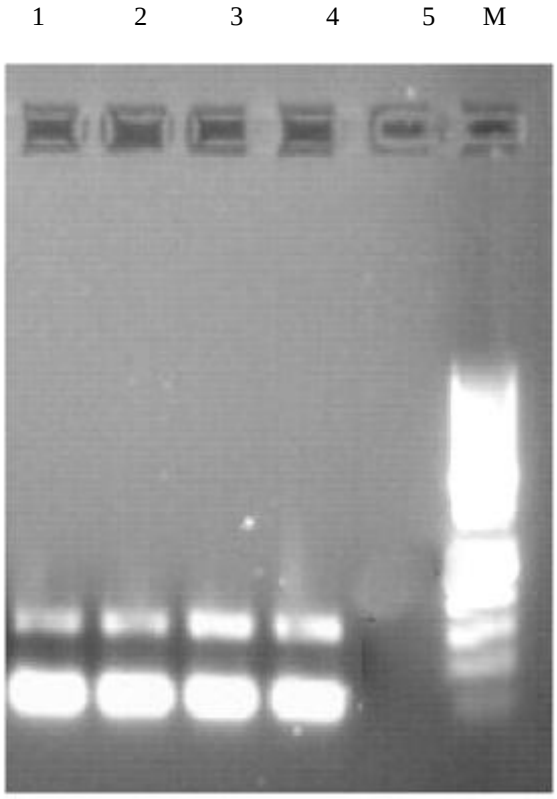

Figure-1 Molecular characterization of probiotic bacteria selected, Lane1-HL1, Lane 2- HL3, Lane 3-HL4, Lane 4-HL5, Lane 5-HL6 and M- Molecular marker