ISOLATION, CHARACTERIZATION AND APPLICATION OF PROBIOTIC ISOLATES FROM GUT REGION OF INDIAN MAJOR CARP.

[1]**Suraj D. Kadam**, [2]Vishwas S. Shembekar, [2]M.A.Dhotre
[1]Assistant Professor, Dept. of Biotechnology, [2]Professor and Head, Dept. of Zoology and Fishery Science, [1]Assistant Professor, [2]Dept. of Biotechnology
[1]Department of Biotechnology,
[1]Rajarshi Shahu Mahavidyalaya,
(Autonomous), Latur (413512), (State) Maharashtra, India.

ABSTRACT

Freshwater aquaculture is continuously expanding and become an important component of Indian fisheries. Disease outburst is one of the major problem facing by aquaculture industries, to overcome this, application of probiotic in fish feed as an alternative to antibiotics is increases in last few decades. Probiotic are good bacteria and thus isolation, characterization and finding new potent probiotic bacteria is a need of sustainable development in this sector. The aim of current work is to isolate, identify and study application of probiotic strains against some common fish pathogens. The species selected for this is Indian major carps (*Catla catla, Labeo rohita, Cirrhinus mirigala*) from Majra dam near to Latur dist. (MH). A total of 15 bacteria were isolated and screened, out of this five strains were selected on the basis of their morphological and biochemical characteristics for further study. These five potent strains were designated as PB1, PB2, PB3, PB4, and PB5. Various growth parameters like pH, temperature and effect of different salt concentration on growth rate was also studied and the result were noted as PB1 and PB2 show growth rate at neutral pH, where other three species show growth at pH 6. Highest viability in the range of 30^0c-40^0c were found and show tolerance at 0.1, 0.2 and 0.3% bile salt concentrations. Antimicrobial activity of all five isolates were evaluated against *Aeromonas hydrophila* using well diffusion method.

Keywords: - Probiotic, Indian major carps, Antimicrobial activity.

I. INTRODUCTION

Probiotics are good bacteria they help to maintain the normal flora and gastrointestinal function of host. This improves the health status and protect against many pathogens by improving and boosting immune system naturally. The term probiotics is defined as "live microbial feed supplements which when administered in adequate amount beneficially affect the host by improving its microbial balance (FAO, 2002).

Fish farming and agriculture are the two major food providers fulfilling major demand of food requirement as the world's population is constantly increasing day by day. By the end of 2050 the world's population may reach to 9 billion, so sustainable development of these two sectors are most important. Increasing population and growing need of healthy food in the demand of today's wold. Fish are rich in many proteins, minerals, and vitamins is one of the good source to fulfil this call. Aquaculture industries are helping to achieve this and making sustainable development of country.

Applications of probiotics as an alternative to antibiotics has been increases in last decades. Disease outburst is major problem with aquaculture industry as water bodies and food is populated with many bacteria some of them are good and some are pathogenic causes various disease in this controlled environment. To overcome this heavy use of chemicals and antibiotics are constantly use in aquaculture industry, but this has many post effect like accumulation of antibiotic in food chain. Looking for an environmentally friendly mode of treatment is use of probiotics.

Therefore isolation and finding of new potent probiotics is a need and currently many researches working on this. Among the many isolates Lactic acid bacteria such as *Lactobacillus sp, Bifidobacterium sp,* and *Enterococcus sp*. are most commonly used as probiotic microorganisms as they are considered as major part of the normal intestinal microflora in animals and humans. It has been well documented in several investigations that lactic acid bacteria are a part of the native microbiota of aquatic animals from temperate regions (Ringo, 2004). It's also well documented that many good bacteria are colonize normally in large intestine and are able to survive in the gastric acid environment as well as bile and pancreatic juice tolerant in the upper small intestine exerting many beneficial effects. (Vieira et al., 2013; Fijan, 2014, Adam et al., 2012, Hyronimus *et al*., 2000; Erkkila and Petaja, 2000).

These bacteria produces many organic acids, proteins which have antimicrobial effect against pathogens in surrounding. Gut region of Indian major carp is a complex ecosystem possessing rich microbiota including many aerobic, facultative anaerobic and obligate anaerobic bacteria (Gomez and Balcazar, 2008). Many review suggest that predominantly aerobes or facultative anaerobes are common microbial sources found in fish digestive tracts many of which are good probiotics. (Bairagi et al., 2002; Saha et al., 2006).

Thus the current work is aim to isolate, enumeration and identification of probiotic bacteria from the gastrointestinal tract of Indian major carp collected from fresh water aquaculture system of Dhanegaon Dam Latur district (MH).

II. MATERIALS AND METHODS

Isolation and identification of probiotics microorganism

Fresh-water Indian major carp fishes *Catla catla, Labeorohita, Cirrhinus mirigala* were collected from Dhanegaon dam located at Latur District, Maharashtra in mid-January 2015 with the help of fisherman net. The fishes were bought into the laboratory and cleaned with sterile distilled water, then for isolation of probiotics fishes were first dissected with the help of sterile scissors. Using sterile distilled water homogenise the digestive tracts and then centrifuge at high speed. Collect supernatant and discard the pellet. Using sterile distilled water serial dilute the supernatant (10^{-2} to 10^{-7}) and then pour on sterile nutrient agar and MRS plate (selective for *Lactobacillus sp*.), incubate at 37^0c for 24 h. observe the colony and subculture for pure culture, characterize on the basis of morphological and biochemical according to Bergey's Manual of Systematic Bacteriology (Whitman et al., 2009).

Study pH, temperature and salt tolerant of isolated species.

The selected species was then used to study properties of probiotics like pH, temperature and salt tolerance. For investigation of pH tolerance media (Nutrient and MRS) using 1N HCl and 1N NaOH ranges from (pH3-pH9) were prepared. (Samelis et al., 1994). Incubate all tubes along with control tube at 37^0c and measure the optical density at 600nm after 0, 2, 4 and 8 h of incubation (Balcázar et al., 2008; Kim and Austin, 2008; Allamesh et al., 2012). Similarly for temperature (20^0c - 80^0c) and bile salt (Oxgall bile salt) (0.1, 0.2 and 0.3% w/v) tolerance tubes were prepare with sterile nutrient broth and MRS broth keeping all other parameter constant.

Antimicrobial activity of the isolated bacterial strains.

A. hydrophila is a common fresh water fish pathogen. A pure culture of *A. hydrophila* was subculture and activated in sterile TSB. All five isolates were also grown and activate using broth. Centrifuge the culture at 8000 rpm for 5 min at 4^0c. Collect the supernatant and use for antimicrobial study. Prepare sterile TSA plate and spread 30 µl *A. hydrophila* culture then using sterile cork borer prepare well of size 50 µl. Using micropipette and sterile tips load the sample in different plates and labelled them. Incubate the plates at 37^0c for 24hrs. Observe the result for zone of inhibition. (Balcázar et al., 2008; Allamesh et al., 2012).

Statistical analysis

Statistical analysis was conducted to compare the quantitative results of treatments using one-way analyses of variance (ANOVA).

III.RESULTS AND DISCUSSION

Isolation and total colony count.

To isolate probiotic bacteria fishes were first dissected and homogenised using sterile distilled water (fig.1) serial dilution and spread the sample over nutrient agar plate to count total number of bacteria. It was observed that gut region of *Catla catla* contain 2.62×10^6 CFU/ml, *Labeo rohita* gut 1.82×10^6 CFU/ml, *Cirrhinus mirigala* gut 1.99×10^6 CFU/ml were populated in intestine. The result in our study was supported by the finding P. Muthukumar in 2015 as *Catla catla* gut 2.72×10^6 CFU/ml, *Labeo rohita*gut 1.87×10^6 CFU/ml, *Cirrhinus mirigala* gut 1.91×10^6 CFU/ml and *Cyprinus carpio* gut 2.19×10^6 CFU/ml of population in intestine of fishes, also Abraham in 2007 and Ringo in 2012 reported similar result suggesting fish intestine is rich and loaded with many different bacteria and have good potential to find out many new probiotic bacteria source.

Fig.1 Isolation of probiotic bacteria from gut region of Indian Major Carp

S.NO.	Fish	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1	*Catla Catla*	4	2	1	2	-
2	Labeo rohita	3	1	3	2	1
3	Cirrhinus mirigala	2	1	2	1	1

Table: 1 Bacterial isolates from intestine of Major Indian carp

The diversity of bacterial population vary from region to region because many factors influence the diversity of microorganisms. In aquaculture all condition are artificially maintained whereas in natural environment the climatic condition affect the growth and diversity of organism. So it depend upon the source from where the species were used for the study.

Morphological and Biochemical analysis for identification of probiotic bacteria.

According to Bergey's Manual of Systematic Bacteriology morphological (Table 1) and biochemical (Table 2) characteristics of all 15 colony were studied. MRS agar is selective media for the isolation of Lactobacillus whereas nutrient broth is generalized media. 5 different Lactobacillus species were selected on MRS and total 10 probiotic species were identified having probiotic potential on nutrient agar media. For further study 5 different bacteria were screen and named as PB1, PB2, PB3, PB4, and PB5. These species then subculture and activated using specialized media. Many study reported that Lactobacillus is common populated probiotic bacteria in fresh water system. In the study of P. Muthukumar 27 different species were isolated and many of them were different strains of Lactobacillus supporting to our findings.

Size	Colony morphology	Colour	Margin	Opacity	Elevation	Motility	Gram Staining/ Cellular morphology	Type of fermentation
Small	Circular	Creamy	Entire	Opaque	Slightly raised	Non-motile	+ve/Rod	Homo Fermentation
Moderate Size	Irregular size	Creamy	Undulate	Translucent	Flat	Motile	+ve/Rod	Hetro Fermentation
Small	Circular	Creamy	Entire	Opaque	raised	Motile	+ve/Rod	Homo Fermentation
Small	Circular	Creamy	Entire	Opaque	raised	Motile	+ve/Rod	Homo Fermentation
Big	Circular	White	Entire	Opaque	raised	Motile	+ve/Rod	Homo Fermentation
Small	Circular	White	Entire	Opaque	convex	Non-motile	+ve/Rod	Homo Fermentation
Moderate Size	Irregular size	White	curled	Opaque	raised	Non-motile	+ve/Rod	Homo Fermentation
Moderate Size	Irregular size	White	curled	Translucent	convex	Motile	+ve/Rod	Homo Fermentation
Big	Elongated	yellow	Erose	Translucent	flat	Motile	+ve/Rod	Homo Fermentation
Big	Circular	yellow	Entire	Opaque	flat	Motile	-ve/Rod	Hetro Fermentation
Big	Circular	yellow	Entire	Opaque	flat	Motile	-ve/Rod	Hetro Fermentation
Small	Circular	Golden yellow	Undulate	Opaque	Slightly raised	Motile	-ve/Rod	Hetro Fermentation
Small	Circular	Creamy	Entire	Opaque	Slightly raised	Motile	-ve/Rod	Hetro Fermentation
Moderate Size	Circular	White	Entire	Opaque	flat	Non-motile	+ve/Rod	Homo Fermentation
Moderate Size	Irregular size	Creamy	Entire	Translucent	Slightly raised	Motile	-ve/Rod	Homo Fermentation

Table: 2 Morphological Characteristics of all isolates

Catalase	oxidase	Urease	Dextrose	Lactose	Fructose	Sucrose	Mannitol	Indol	H2S Production	MR/VP	Citrate Utilization	Starch hydrolysis
-	-	-	+(no gas)	-	+	+	+	-	+	+/-	+	+
+	-	-	+(no gas)	-	+	+	+	+	-	+/+	-	+
+	-	-	+(no gas)	-	+	+	+	+	-	+/+	+	+
+	-	-	+(no gas)	-	+	+	+	+	-	+/+	+	-
+	-	-	+(no gas)	-	+	+	+	+	-	+/+	+	+
+	+	-	+(no gas)	-	+	+	+	-	-	+/+	+	+
-	-	+	-	-	+	+	-	-	-	+/+	-	+
-	-	-	-no gas)	+	+	+	+	+	-	+/+	-	-
-	-	-	+(no gas)	+	+	+	+	+	-	-/+	+	+
+	+	+	+(no gas)	-	+	-	-	+	-	+/+	+	+
+	-	-	+(no gas)	+	+	+	-	+	-	+/+	+	+
+	-	-	+(no gas)	-	+	+	+	+	+	+/+	+	+
+	+	+	+(no gas)	-	+	+	+	+	+	+/+	+	-
-	-	-	+(no gas)	-	+	+	+	-	+	+/-	+	+
+	-	-	+(no gas)	-	+	+	+	-	+	+/+	+	+

Table: 3 Biochemical Characteristics of all isolates.

Characteristics	PB1	PB2	PB3	PB4	PB5
Colony morphology	White colour, Smooth, irregular shape	Small white,	Cream colour, Smooth, circular e	Smooth, circular	White colour, Smooth, irregular shape
Gram Staining/ Cellular morphology	+ve, rod, non motile.	+ve, rod, non motile.	+ve, rod, non motile.	+ve, rod, non motile.	+ve, rod, non motile.
Catalase	-	+	-	+	-
oxidase	-	+	-	+	-
Urease	-	+	-	+	-
Dextrose	+	+	+	+	+
Lactose	+	+	+	+	+
Fructose	+	+	+	+	+
Sucrose	+	+	+	+	+
Mannitol	+	+	+	+	+
Indol	+	-	+	+	+
H2S Production	+	+	+	+	+

MR/VP	+	+	+	+	+
Citrate Utilization	-	-	-	-	-
-Starch hydrolysis	-	-	-	-	-
Possible Microorganism	*Lactobacillus acidophilus*	*Bacillus spp.*	*Lactobacillus plantarum*	*Bacillus spp.*	*Lactobacillus casei*

Table: 4 Five selected species for further study.

Effect of pH, Temperature, and Bile salt on the growth of isolates.

Probiotics are good bacteria they maintain healthy environment, secrets many essential enzyme and regulate the metabolic process. These bacteria are mostly pH and salt tolerant. In the current study we have studied probiotic potential of all five selected bacteria and the result were noted down. Viable cells were counted at different time interval 0,1,2,3 and 4. And the result showed that out of five selected species PB1, PB4, and PB5 show highest growth at pH range 3-6 at 37^0c where as other two strains showed highest growth at pH range from 6 to 8. According to Mohammed A. Ramadan et. Al. in 2014 concluded in their research that Lactobacillus plantarum show highest acid tolerance among all other isolates. In the study of Sahadeva et.al. (2011) different strains in different commercial products show good tolerance to pH 3.0 and 7.2.

All five bacteria show growth at low pH 3.0 indicating ability of probiotic as stress tolerance (fig.2), one of the important property of probiotic. According to the finding of Prasad et.al. 1998, Haddadin et.al. 2004 and Liong et.al. 2005 stated that resistance at pH 3 is consider as a standard for acid tolerance of probiotic culture.

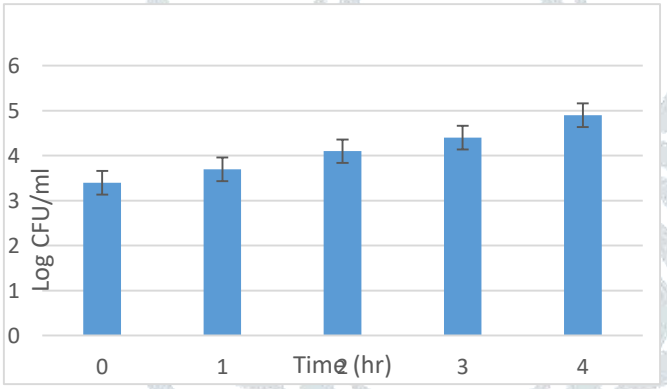

Figure 2 Resistance of Probiotic isolates at acidic pH (3)

In the study of Hassan (2012) reported stress tolerance test at pH level 2.0 and 3.0 while keeping 7.5 pH as control, in the current study below pH 3.0 no. of colony was observe in decreasing range. In the study reported by Chan *et.al*.in 2011 low pH inhibit the growth and viability of bacteria by affecting their metabolism activities, this finding was also supported by Prasad *et. al*. in 1998 and *Delgado et.al.* in 2007. At high pH 9.0 and above no growth was observe that means this is adverse pH for the growth of bacteria.

Temperature ranges from 30^0c-40^0c were found and optimum temperature range for the growth of all five isolates. Maximum growth was obtained at 37^0c temp. Bile salt tolerance is an important property of a probiotic isolation and selection, in current work all five selected species show better tolerance to bile salt at various concentration i.e. 0.0(control) 0.1, 0.2 and 0.3%. In general high bile salt concentration cause cellular haemostasis disruption which leads to dissociation of lipid bilayer and integral proteins of cell membrane, resulting in leakage of bacterial content and the result is cell death. The result show decrees in cellular count as bile salt cons. increases and decreases in pH. Similar finding was observed in the study of Sahadeva et.al. (2011).

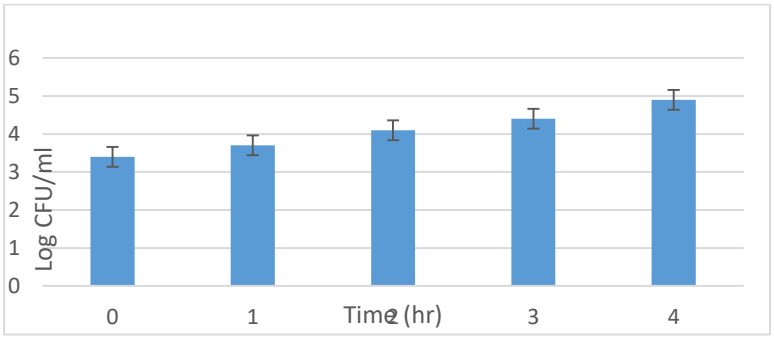

Figure 3 Effect of bile salt on the growth of selected probiotic isolates.

Out of 5 isolates PB1 and PB4 showed better tolerance to bile salt at highest concentration (fig3). Similar finding was observe in the study of Hassan et.al. 2012 where, out of 27 isolates most of them did not show resistance to acid and bile only *Lactobacillus casei* show resistance to acid and bile salt condition. On the basis of phenotype and biochemical characteristics PB1 and PB4 show characteristics similar to *Lactobacillus casei and L. plantarum* so supporting to our findings and thus we can consider this is as potent probiotic but needed further investigation at molecular level. Bile salt tolerance is one of the most important property of probiotics, this increases the ability of bacteria to grow and survive in the intestine of fish. Therefore this is one of source of interest to isolate potent probiotic of many commercial and wild type aquatic animals. Similar finding was recorded in the study of Salminen et al., 2004. In our study 3 bacteria show maximum tolerance i.e. at 0.3% cons. of which was found related with the findings Kim and Austin (2008) and Cebeci et al. (2003). Thus this help in identification and screen as probiotic bacteria for further study.

To study antibacterial activity by well diffusion method.

Aeromonas hydrophila a common fish pathogen was used to check effect of antibacterial activity of isolates. PB1 show highest activity against the pathogen with zone of inhibition 15 mm followed by PB3, PB2, PB4 and PB5 show zone of inhibition 13mm, 12mm,11mm and 11mm respectively (Table 5)

Sr. No.	Isolates	Diameter of the zone of Inhibition against *Aeromonas hydrophila* (mm)
1	PB1	15
2	PB2	12
3	PB3	13
4	PB4	11
5	PB5	11
6	Positive Control standard antibiotic	13
7	Negative control (water)	-

Table: 5 Antimicrobial activity of Isolates against *Aeromonas hydrophila* (common fish pathogen)

According to report published by Shubhadeep Ghosh et.al. (2007) two isolates namely SG1 and SG4 from the strains *C. mrigala* had zone of inhibition against *A. hydrophila* (4.05±1.73), *P. fluorescens* (5.21±0.57) and *E. tarda* (3.8±1.27). Another significant study carried out by Aly et al. (2008) and Rengpipat et al. (2008) show zone of inhibition against *A. hydrophila* when a probiotic applications was used. A similar frequency of inhibitory result was observe in the study of Kaynar and Beyatli (2012), different species of Bacillus show significant zone of inhibition against common pathogen *P. fluorescens.* In our study all the isolated show activity against *A. hydrophila* indicating a good alternative to antibiotics hence, we can conclude that probiotics is safe and eco-friendly approach toward sustainable development of aquaculture industry it can reduce the cost with suitable use of probiotic as a good alternative to chemicals.

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