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Project Completion Report of a Minor Research Project on

"Microbial diversity and ecology in gut ecosystem of Indian Major Carps of Dnyaneshwar reservoir, Rahuri"

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Table of Contents

Introduction

India is endowed with bounty of varied climatic condition, microbial diversity and fish fauna and aquaculture systems. India ranked third in global fish production. India trades about 2.4% of global fish market with annual export earning over US$1311 million. Aquaculture has emerged as the fastest growing food producing sector and developed as an important component in food security. For sustained and enhanced productivity, health management strategies must go beyond the use of antibiotics and chemotherapeutics which creates resistant bacteria and immunosuppression in the host. The adverse effect of antibiotics is caused by their influence on the aquatic microflora and the retention of harmful residues in aquatic animals (Panigrahi and Azad, 2007).

The Indian subcontinent is at the confluence of three biogeographic realms, viz., the Palaearctic, Afro-Tropical and Indo-Malayan which exhibits a great variety of ecological habitats, harbouring rich ichthyofaunal diversity. The Indian species represent about 8.9% of the known fish species of the world (Devi and Indra, ZSI, India). The major carps of India fall under three genera, *Catla*, *Labeo* and *Cirrhinus*. *Catla catala* is Zooplanktophagous whereas *Cirrhinus mrigala* is detritivorous. *Labeo rohita* is omnivorous but mostly feed on plant matter (Bairagi et al., 2002). Taking advantage of the mutually compatible and complimentary food habits of these carps, the present day intensive and extensive multispecies culture has been developed from 1970s, which is popularly known as the composite carp culture. This technology has developed and demonstrated a gradual increase in the fish production from the culture systems from 3–4 t/ha/year to 10–15 t/ha/year (FAO, 2011).

As in all vertebrates, Fishes also harbour microbial population on eggs, skin, gills & intestine (Cahill, 1990). Microflora of skin & gills may be transient. Microbial flora of intestine is dense & dynamic than surrounding which implies that intestine provides favourable ecological niches for these microorganisms (Austin and Austin, 1999). Intestine is a multifunctional organ, having role in digestion & absorption of food, electrolyte balance, endocrine regulation of food metabolism, immunity against pathogens. It is also a potential route for pathogen to invade and infect. The microorganisms in gastrointestinal (GI) tract give protection to host by production of inhibitory chemicals and competition for food & space. They are also found to influence the expression of genes involves in epithelial proliferation, metabolism and innate immunity (Rawls et al., 2004). For different experimental purposes, microbial gut flora has been studied by several workers: microbial spoilage (Joseph et al. 1988); relation between environment and fish

microflora (Horsely et al. 1973); monitoring change in fish form (Allen et al. 1983); microbial flora as food of fish (Kamjunke et al. 2002); microbial flora for production of enzymes (Bairagi et al. 2002); antibiotic resistance profiles of indigenous flora (Spanggard et al. 2000).

The microflora of digestive tract has been surveyed by many researchers using conventional culture dependent techniques. It was demonstrated that fish possesses specific intestinal microbiota consisting of aerobic, facultative anaerobic and obligate anaerobic bacteria (Cahill, 1990; Ringo et al. 1995, 2003). But bacterial composition varies with age, individuals, nutritional status, environmental condition and complexity of fish digestive system. Bandopadhyay et al. (2006) have studied intestinal microflora of farm raised Indian Major Carps by conventional methods. Ghosh et al. (2007) have isolated some probiotic strains by conventional culture dependent methods form juveniles and fingerlings of Indian Major Carps cultured in sewage fed pond. Ray et al. (2009) identified the gut associated amylase, cellulase and protease producing bacteria only by both conventional and molecular methods.

The molecular methods have been generated advances in microbial ecology of gastrointestinal tract that has not been achieved by the use of conventional techniques. But the role of classical microbial ecology should not be underestimated. The molecular methods make the use of information originating from microorganisms which are cultured and deposited in various repositories and form a small fraction of biodiversity in nature. The lack of resolving power limits the inferential value of molecular techniques at the level of species, the most important unit for expressing biodiversity (Palleroni, 1997). Molecular approaches should have an important role as guides for isolation and characterization of prokaryotic biodiversity. Thus culture based techniques should be used in combination with new molecular techniques to improve cultivation, speciation and study biodiversity. Therefore present study is to be conducted combining molecular measures of species composition and abundance with measurement of biochemical activity to determine precise role or function that a specific organism plays in its natural environment and its quantitative contribution to the whole.

Study Area: Dnyaneshwar Reservoir is a dam built across Mula River at Rahuri District Ahmednagar in Maharashtra state, India. The dam is located at latitude 19^0- 20' to 19^0- 35' N and longitude 74^0- 25' to 74^0- 36' E. The water storage capacity of the dam is 26 TMC. It experiences an average rain fall 58 cm. Maximum depth is 67.97 m. The reservoir bottom is composed of detritus-mud layer in the littoral zone. The dam water has been used for drinking and irrigation by the people of Ahmednagar city and districts (Dams of India, Ahmednagar gazetteers).

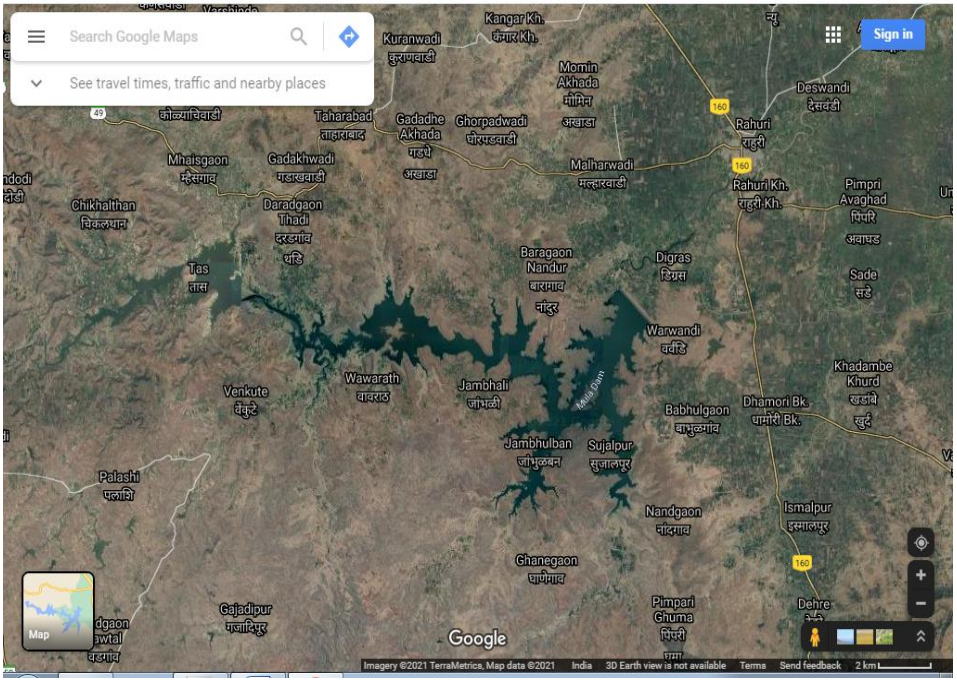

Fig 1: Satellite Picture of Mula Dam (Source - Google Map)

Significance of the study: Ih is generally accepted that conventional culture based techniques do not represent correct picture of bacterial diversity in fish gut. But to present more reliable information on gut microbiota of fish, molecular approaches should be used in combination. The information regarding intestinal microbial flora in fish and their ecology is rare. However there is information regarding endogenous digestive enzymes in fish. Generally bacteria are abundant in the environment in which fish live and it is avoidable them being a component of their diet. In present investigation considerable microbial population may be found in GI tracts of the fishes and certain strains may have symbiotic association with the host playing a role in their digestion by producing various extracellular enzymes. So such strains, after strategic screening can be used as probiotic in formulating cost effective fish diet.

1. Present study will help to identify the intestinal microbial diversity of Indian Major Carps from Western Ghats for the first time.
2. This study will also reveals about water quality of reservoir and quality of fishes as food.
3. Knowledge about the fish microbiota will help to understand the disturbances, if there are any, brought about during the disease outbreaks. Hence it has great importance.

4. In present study, an attempt will be made to investigate the enzyme producing microflora in the gut of Indian Major Carps.
5. This work will investigate the relationships between the gastrointestinal bacteria and host fishes.
6. The information will facilitate the formulation of effective probionts from Indian Major Carps.
7. This work might have valuable implications for management practices in aquaculture.

Aims and objectives

1. To study the microbial diversity in gut environment of Indian Major Carps.
2. To assign the functional roles to these microorganisms.
3. To assess their significance and contribution to gut environment of Indian Major Carps.
4. To screen isolates for their potential as probiotic.

Materials and Methods

1. **Sampling of Water & Physico – Chemical analysis:** Sampling for the physico -chemical parameters was carried out for pre-monsoon and post-monsoon period for 2014-2015. As per the norms of the APHA, the water samples were to be collected in plastic bottles and partially to be tested in the field, as well as in the laboratories.
2. **Sampling of Fishes:** The adult Indian Major Carps (*Labeo rohita*, *Catla catla*, *Cirrhinus mrigala*) fishes were sampled randomly from study area during the same period of water sampling. The fish samples were transported to the laboratory in live condition. The feeding habits, sex, total length (T_L), average weight (g) and gut length (L_G) were recorded. The relative gut length (L_G/T_L) was then calculated.
3. **Isolation of Culturable Intestinal Bacteria**: The fishes were starved for 24 h to make their intestinal tract clear and eliminate the bacteria that are transit in nature. After starvation, they were sacrificed and GI tract to be removed. A homogenate solution of GI tracts can be made with sterile saline solution. It is then serially diluted and plated in triplicates on sterile Tryptone soya agar (TSA) aseptically. Then plates were incubated in order to determine the aerobic plate count. The isolates were maintained in pure form.
4. **Screening of isolates for extracellular qualitative enzyme production**: The isolates screened for qualitative enzyme production of extracellular amylase, cellulase, lipase and protease. This information would facilitate the formulation of cost effective probiotic incorporated in fish feed.
5. **Morphological & Biochemical Characterization of Culturable Intestinal Bacteria:** Characterization and identification of isolates was done based on Gram reaction; spore formation; cellular morphology; motility; growth in different salinities and pH; sugar utilization; amino acid decarboxylation; catalase and oxidase production; nitrate reduction; hydrogen sulfide production; starch, casein, and urea hydrolysis; gelatin liquefaction; and IMVIC tests.
6. **DNA Extraction, PCR Amplification and DNA sequencing of cultured Bacteria from intestine samples:** The culturable bacterial isolates were used for DNA extraction and PCR amplification of 16s rDNA using universal primer following its sequencing.
7. **Direct DNA Extraction, PCR Amplification and DNA sequencing of from intestine samples:** The intestinal mucous was collected from same fishes and to be used for DNA extraction. The 16s rDNA was amplified using universal primer following its sequencing.

8. **Sequence Analysis:** 16s rDNA sequences were checked for chimeric constructs using the CHECK_CHIMERA program of ribosomal database project (RDP) website (http://rdp.cme.msu.edu). The sequence similarity analysis carried out using bioinformatics tools. The phylogenetic relationship was then established.

Results and Discussion

The values of physicochemical parameters of the water samples obtained in pre and post mason period from Dnyaneshwar reservoir, Rahuri shows that water is potable and there in no addition of sewage. The water is of good quality (Table 1).

Table 1: Physico-Chemical parameters of water samples

Sr. No.	Parameters	Pre-mansoon			Post-manson			WHO Permissible Limit
		Site I	Site II	Site III	Site I	Site II	Site III	
1.	Temperature	29.5	29.4	29.3	27.1	27.2	27.3	-
2.	pH	7.0	7.2	7.1	7.5	7.6	7.4	6.5-9.2
3.	Alkalinity	130	125	140	100	104	108	200 mg/L
4.	Chlorides	51.65	60.06	60.09	97.05	92.02	95.00	200mg/L
5.	Magnesium	15.93	14.80	12.30	14.40	18.06	20.11	30 mg/L
6.	Total Hardness as $CaCO_3$	75	80	85	100	88	92	500 mg/L
7.	Calcium Hardness	55.6	52.4	40.9	52.4	40.1	38.9	75mg/L
8.	Magnesium Hardness	24.4	34.7	43.5	48.6	47.7	48.1	50 mg/L
9.	Nitrate	2.0	2.6	2.4	2.8	2.7	2.9	45 mg/L
10.	Phosphate	0.78	0.84	0.80	1.51	1.35	1.25	0.01-1.0mg/L

After analysis of food habits, average total length, average weight, average gut length, relative gut length and bacterial flora (Table 2 and 3) of Indian Major Carps, shows that aerobic population is maximum in *Catla catla* followed by *Cirrhinus mrigala* and *Labeo rohita* due to their feeding habit and habitat in the reservoir. The extracellular enzyme production abilities of the isolates were assessed qualitatively (Table 4). Amylase activity was shown by LR3, CC1, CC2, CC3, CM1 and CM3 whereas cellulase activity was showed by LR3, CC1, CC2 and CM3. Lipase activity was present in LR1, LR2, CC1 and CM3 while Protease activity was present in most of isolates except LR3 and CM1.

The morphological and biochemical characterization of the isolates of bacterial strains from GI tracts (Table 5) and their identification with partial sequence of 16s rDNA from BLAST search in GenBank revealed the dominance of gram negative rod shaped bacteria capable of producing various hydrolytic enzymes. The genera like Aeromonas, *Bacillus, Vibrio, Micrococcus, Flavobacterium, Pseudomonas, Moraxella,* etc. are seems to be those which can survive and multiply in the Gi tract of Indian Major Carps from Dnyaneshwar Reservoir, Rahuri. Results of present experimental work might have a valuable implication in the aquaculture practices by using these gut microflora.

Table 2: Food habits, average total length, average weight, average gut length and relative gut length of Indian Major Carps

Fish Species	Food Habitat	Sex	Average Total length (T_L) (cm)	Average weight (g)	Average Gut length (L_G) (cm)	Relative Gut length (L_G/T_L)
Labeo rohita	Herbivorous	Male	**15.4**	**250.8**	**140.5**	**09.12**
		Female	**14.8**	**240.4**	**130.4**	**08.81**
Catla catla	Omnivorous	Male	**18.4**	**210.5**	**206.0**	**11.19**
		Female	**18.2**	**205.4**	**189.3**	**10.40**
Cirrhinus mrigala	Detritivorous	Male	**17.4**	**160.4**	**130.7**	**07.79**
		Female	**16.8**	**150.2**	**126**	**07.50**

Table 3: Aerobic heterotrophic bacterial count in fish GI tracts

Fish Species	Food Habitat	Average Bacterial Populations (CFU g^{-1} GI tract) on TSA Plate ($x10^7$)
Labeo rohita	Herbivorous	2.6
Catla catla	Omnivorous	3.2
Cirrhinus mrigala	Detritivorous	2.9

Table 4: Qualitative extracellular enzyme producing abilities of bacterial isolates from GI tracts of three fish species

Fish Species	Isolates Strain No.	Enzyme Producing Capacity#			
		Amylase	Cellulase	Lipase	Protease
Labeo rohita	LR1	-	-	+	+
	LR2	-	-	+	+
	LR3	+	+	-	-
Catla catla	CC1	+	+	+	+
	CC2	+	+	-	+
	CC3	+	ND	-	+
Cirrhinus mrigala	CM1	+	-	-	-
	CM2	ND	ND		+
	CM3	+	+	+	+

With pure culture of GI tracts

NA = Not detected, + and - sign indicates ability and inability to produce the extracellular enzyme

Table 5: Morphological & biochemical characterization of culturable intestinal bacterial Isolates

Parameter	**Characteristics**					
	LR1	**LR2**	**CC1**	**CC2**	**CM1**	**CM3**
Gram reaction **1. Shape** **2. Size**	- Rod, Short chain Moderate	- Rod, Short chain Moderate	- Rod, Short chain Moderate	 Coccobacilli, Moderate	- Vibrio, Single/pairs Moderate	- Rod Single/pairs Moderate
Spore formation **1. Endospore** **2. Shape**	 + Terminal	 + Terminal	 + Terminal	 + Terminal	 + Terminal	 + Terminal
Cellular morphology **1. Configuration** **2. Elevations** **3. Surface** **4. Pigments**	 Round Convex Mucosal ND	 Round Convex Mucosal +	 Round Convex Mucosal ND	 	 Round Flat Shiny -	 Round Flat Shiny +
Motility	+	+	+	-	+	-
Growth in different pH **1.** pH 5-9 **2.** pH 11	 + ND	 + ND	 + +	 ND ND	 + ND	 + ND
Growth in different salinities **1.** NaCL 2-9 % **2.** 10%	 + -	 + -	 + +	 ND	 	 + ND
Sugar utilization **1.** Maltose **2.** Dextrose	 + +	 + +	 ND +	 - -	 + +	 - -
Catalase	+	+	+	+	+	ND
Oxidase production	+	+	+	+	+	+
Nitrate reduction	+	+	ND	-	+	+
H_2S production	-	-	ND	-	-	-
Starch hydrolysis	+	+	ND	+	-	ND
Casein hydrolysis	ND	ND	+	-	ND	ND
Urea hydrolysis	-	-	ND	-	-	-
Gelatin liquefaction	+	+	+	-	+	+
Indole	+	_	ND	-	+	-
Citrate	+	+	+	-	+	-
VP	-	-	-	-	+	-
Candidate Species	*Aeromonas spp.*	*Pseudomonas spp.*	*Bacillus spp.*	*Moraxella spp.*	*Vibrio spp.*	*Flavobacterium spp.*

+ = Positive, - = Negative, ND = Not detected

Table 6: Identification of bacterial strains from GI tracts with partial sequence of 16s rDNA from BLAST search

Isolates Strain No.	**Closest Relative**	**Similarity (%)**
LR1	*Aeromonas spp.*	**98**
LR2	*Pseudomonas spp.*	**96**
CC1	*Bacillus spp.*	**95**
CC2	*Moraxella spp.*	**93**
CM1	*Vibrio spp.*	**97**
CM3	*Flavobacterium spp.*	**92**

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