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Microbial diversity and enzyme production in mullet *Mugil cephalus* L. (Pisces) along Goa, west coast of India

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Twelve bacterial species were isolated from different organs of the mullets. A comparative analysis of different bacterial communities isolated from surface, gills and intestinal tract showed significantly diverse population with reference to different groups and density. During present investigations, some of the important microbial groups occurred in the different regions of the *Mugil cephalus* were *Pseudomonas* sp., *Vibrio parahaemolyticus*, *Serratia* sp., *Azotobacter* sp., *Streptococcus* sp., *Proteus vulgaris*, *Planococcus* sp., *Bacillus subtilis* and *Serratia marescens*. Further study on production of enzyme in the gut content suggested that proteolytic and amylolytic activity were of elevated level, thus emphasizing that these microbial communities do play an important role in promoting efficient food utilization among mullets.

[**Key words**: Mullets, intestinal micro-flora, proteolytic and amylolytic enzyme, microbial diversity, enzymes, *Mugil cephalus*]

Introduction

Microbes act as probiotics through antibacterial activity in the intestine and enhance growth of phytoplankton in presence of marine bacteria [1,2] thus enhancing the efficient utilization of the available resources through acceleration of mineralization process. Further, microbial diversity in marine environment is an important entity that maintains ecological balance through various microbial processes [3] and enhance fertility of ponds [4]. Aquatic ecosystem although harbors a sizable population of microbes [5,6] are often considered as an index of water quality. These ubiquitous microorganisms do find various surfaces or organs of aquatic organisms for colonization. The present study provides an account of microbial communities isolated from surface, gills and intestinal tract of *Mugil cephalus*. Further, an attempt has been made to study production of different enzymes such as proteolytic, amylolytic, and cellulases by the isolated intestinal micro-flora of the mullets as these studies will provide better insight about the possible role of these microbial communities to enhance the efficiency of food utilization through the enzymatic breakdown of macromolecules.

Materials and Methods

The estuarine marshy region of Goa occupy an important place with reference to the fish resources as these areas produce substantial quantity of organic matter thus enhancing secondary production. The present area of study (lat 15° 29' to 15° 33' N and long 73° 45' to 73° 59' E) forms an important region with reference to exploitation of mullets. The harvesting of mullets was done by cast net, pole and line and gill nets. During the period of present study (July – December, 2002) the gray mullets (30 – 40 individuals) in the size range of 90-200 mm were obtained from the sampling site at fortnightly interval.

The collection of fish sample for the assessment of microbial diversity was done using cast net and were aseptically obtained and transferred to laboratory under sterile conditions. The harboring of microbial population from different organs (surface, gills and intestinal tract) was done in triplicate by swabbing the fish using sterile cotton under aseptic conditions in the laboratory. Subsequently, the swabbed cotton was dipped into sterile saline to release microbial content. The sample along with the saline was used for further microbial studies. The isolation of the bacterial cultures was carried using serial dilution technique. The serially diluted contents were then streaked on plates with nutrient agar medium incubated at room temperature for 48 hr. The isolated colonies obtained were then transferred on the slants and the culture was maintained for further studies. The cultures obtained were subjected to morphological and biochemical

Table 1 — Morphological and biochemical tests performed for the identification of isolated bacterial strains from the surface of the mullets

Tests	Species					
	1	2	3	4	5	6
Size	1mm	Pinpoint	0.1 mm	1 mm	0.2 mm	0.1 mm
Shape	Circular	Circular	Circular	Circular	Circular	Circular
Color	Opalescent	Creamy white	Buff	Orange	White	Cream
Elevation	Raised	Convex	Convex	Raised	Raised	Convex
Gram stain	Gram –ve coccobacilli	Gram –ve curved rods	Gram –ve coccobacilli	Gram –ve coccobacilli	Gram –ve cocci	Gram –ve staright rods
Motility	Motile	Motile	Motile	Motile	Motile	Motile
Aerobic growth	Aerobic	Aerobic	Facultative aerobic	Aerobic	Aerobic	Facultative anaerobic
Oxidase test	+	+	=	=	=	=
Catalase test	+	+	+	+	=	+
Utilization of CHO						
Glucose	+	+	+	+	+	+
Lactose	=	=	=	=	=	+
Maltose	=	+	+	+	+	+
Mannitol	+	+	+	+	+	+
Xylose	=	=	=	=	=	+
Sorbitol	=	+	=	+	+	+
Sucrose	=	=	+	+	+	+
Fructose	=	=	+	=	=	=
Indole production	=	=	+	+	=	=
Methyl red test	=	=	=	=	+	=
Voges-Prosauer test	=	=	+	+	=	+
Citric acid	=	+	=	+	+	+
Nitrate reduction	=	=	=	=	+	=
Urease test	=	=	=	=	=	+
H_2S production	=	=	=	=	=	=
Lipolytic activity	=	=	=	=	=	=
Gelatin liquefaction	+	+	+	+	=	=
Salt tolerance test (Nacl)						
0%	=	=	=	=	=	=
0,5%	=	+	=	=	=	=
3%	=	+	=	=	=	=
6%	=	+	+	=	=	=
8%	=	+	+	=	=	=
Arginine decarboxylase	+	=	=	=	+	+
Lysin decarboxylase	+	+	+	+	=	=
Ornithine decarboxylase	=	=	=	+	=	+
Sensitivity to 0/129	=	+	=	=	=	=
	Pseudomonas Spp.	*Vibrio* Spp.	*Aeromonas* Spp.	*Serratia* Spp.	*Azotobacter* Spp.	*Enterobacter* Spp.

Table 2 — Morphological and biochemical tests for taxonomic studies of bacterial cultures isolated from gills of fish

Tests	Species				
	1	2	3	4	5
Size	1mm	0.2 mm	0.2 mm	2 mm	Pinpoint
Shape	Circular	Circular	Circular	Circular	Circular
Color	Opalescent	White	White	White	Orange
Elevation	Raised	Raised	Raised	Flat	Convex
Gram stain	Gram –ve	Gram –ve	Gram –ve	Gram –ve	Gram +ve
	Coccobacilli	cocci	cocci	coccobacilli	cocci
Motility	Motile	Motile	Non-motile	Motile	Motile
Aerobic growth	Aerobic	Aerobic	Facultative anaerobic	Anaerobic	Aerobic
Oxidase test	+	=	=	=	=
Catalase test	+	=	=	+	+
Utilization of CHO					
Glucose	+	+	+	+	+
Lactose	+	+	=	=	+
Maltose	=	+	+	+	+
Mannitol	+	+	=	=	+
Xylose	=	=	+	+	=
Sorbitol	=	+	=	=	=
Sucrose	=	+	=	+	+
Fructose	=	=	=	=	=
Indole production	=	=	=	=	=
Methyl red test	=	+	+	+	=
Voges-Prosauer test	=	=	=	+	=
Citric acid	=	+	=	+	+
Nitrate reduction	=	+	=	+	=
Urease test	=	=	=	+	=
NH_4 production to arginine	=	=	=	+	=
H_2S production	=	=	=	+	=
Lipolytic activity	=	=	=	+	=
Gelatin liquefaction	+	=	=	+	+
Salt tolerance test (Nacl)					
0%	=	=	=	=	=
0,5%	=	=	=	=	=
3%	=	=	=	=	=
6%	+	=	=	=	=
8%	+	+	=	=	=
Arginine decarboxylase	+	=	=	=	=
Lysin decarboxylase	+	=	=	=	=
Ornithine decarboxylase	=	=	=	=	=
Sensitivity to 0/129	=	=	=	=	=
	Pseudomonas Spp.	*Azotobacter* Spp.	*Streptococcuss* Spp.	*Proteus vulgaris*.	*Planococcusr* Spp.

Table 3 — Morphological and biochemical tests performed for the identification of isolated bacterial strains from the intestinal tract of the mullets

Tests	Species							
	1	2	3	4	5	6	7	8
Size	1mm	Pinpoint	Pinpoint	2 mm	1 mm	1 mm	0.1 mm	0.2 mm
Shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Color	Opalescent	Creamy white Creamy white White	White	Orange	Cream	White		
Elevation	Raised	Convex	Convex	Flat	Raised	Flat	Convex	Raised
Gram stain	Gram –ve coccobacilli	Gram –ve rods	Gram –ve curved rods	Gram –ve coccobacilli	Gram –ve bacilli	Gram –ve coccobacilli	Gram –ve straight rods	Gram +ve cocci
Motility	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Non-motile
Aerobic growth	Aerobic anaerobic	Facultative anaerobic	Aerobic anaerobic	Anaerobic	Aerobic	Aerobic	Facultative	Facultative
Oxidase test	+	+	+	=	=	=	=	=
Catalase test	+	+	+	+	+	+	+	=
Utilization of CHO								
Glucose	+	+	+	+	+	+	+	+
Lactose	=	=	=	=	=	=	=	+
Maltose	=	+	+	+	+	+	+	+
Mannitol	+	+	+	=	=	+	+	=
Xylose	=	=	=	+	+	=	+	+
Sorbitol	=	=	+	=	=	+	+	=
Sucrose	=	+	=	+	=	+	+	=
Fructose	=	=	=	=	+	+	=	=
Indole production	=	=	=	=	+	+	=	=
Methyl red test	=	=	=	+	=	=	=	+
Voges-Prosauer test	=	+	=	+	=	+	+	=
Citric acid	=	+	+	+	=	+	+	=
Nitrate reduction	=	=	=	+	+	+	=	=
Urease test	+	+	+	+	+	=	+	=
NH_4 production to arginine	=	=	=	+	=	=	=	=
H_2S production	=	=	=	+	=	=	=	+
Lipolytic activity	=	+	+	+	=	=	+	=
Gelatin liquefaction	+	=	+	+	=	+	=	=
Salt tolerance test (Nacl)								
0%	=	=	=	=	=	=	=	=
0,5%	=	+	+	=	=	=	=	=
3%	=	+	+	=	=	=	=	=
6%	=	+	+	=	=	=	=	=
8%	+	+	+	=	=	=	+	+
Arginine decarboxylase	+	=	=	=	=	=	=	=
Lysin decarboxylase	=	+	+	=	=	+	+	=
Ornithine decarboxylase	=	+	+	=	=	+	+	=
Sensitivity to 0/129	=	+	+	=	=	=	=	=
	Pseudomonas Spp.	*Vibrio parahaemolyticus*	*Vibrio* Spp.	*Proteus vulgaris*	*Bacillus subtilis*	*Serratia marcescens*	*Enterobacter* Spp.	*Streptococcus* Spp.

characteristics [7,8] so as to enable the identification of the culture. The morphological tests performed were size, shape, color and elevation of the colonies, whereas other tests such as gram staining of cells, motility, aerobic / anaerobic were also carried out. The standard biochemical tests used to identify isolated cultures were fermentation of sugars, hydrolysis of starch, production of indole, methyl red test, Vogues Proskauer test, citric acid test, urease test, nitrate reduction test, gelatin liquefaction, catalase test, Vibrio static test, etc. In addition to the above mentioned biochemical tests the ability of the isolated bacterial cultures to produce enzymes such as amylase, protease, lipases and cellulases were explored using the following strategy.

Proteolytic micro-organisms were isolated by inoculating the bacteria from the gut content on skimmed milk agar. The plates were incubated at room temperature for 72 hr. Growth of proteolytic microorganisms and production of extra-cellular enzymes protease was detected by appearance of haloes or clearings around the colonies. The bacterial flora from the gut was inoculated on starch agar plate for the isolation of amylolytic microorganisms. The plates were incubated at room temperature at 72 hrs. These plates were then flooded with iodine solution. The starch hydrolyzing enzyme amylase produced by amylolytic micro-organisms hydrolyzed the starch in the medium producing haloes were observed on flooding the plate with iodine. The clearings around the colonies due to starch hydrolysis remained unstained while the remaining portion of the plate was blue in color.

Carboxy Methyl Cellulose (CMC) agar plate was used for the isolation of cellulolytic microorganisms. The plates were incubated at room temperature for 72 hr. These were then flooded with 0.1 % Congo red solution. The cellulolytic microorganisms hydrolyzed the cellulose in the medium producing haloes were observed on flooding the plate with 0.1% Congo red solution.

Results and Discussion

Studies on the microbial communities isolated from different organs (surface, gills and intestinal tract) of mullet collected from the study area showed a highly diverse and varied microbial population associated with different organs. The characteristics of different bacterial cultures isolated from the surface of the mullet using morphological and biochemical tests are given in Table 1. During the present study, a total of six bacterial strains were isolated from the surface swabs of the mullets. The bacterial strains isolated were represented by *Serratia* spp., *Azotobacter* spp., *Pseudomonas* spp., *Vibrio* spp, *Aeromonas* spp., and *Enterobacter* spp. Most of the bacterial communities isolated in the present study also form an endemic flora of the surrounding medium. Rivonker *et al.*[8] isolated and characterized chitin degrading bacteria among the wild prawns collected from Goa. Earlier studies [4,9] suggest that the microbial communities associated with the external surfaces depend significantly on the environment of the host. Although, the fish samples were obtained during monsoon season, there was no occurrence of terrestrial microbial flora such as *Bacillus* spp., *Flavobacterium* spp., etc. associated with fish surfaces. Austin[6] conducted studies on bacterial flora associated with surfaces of healthy fish and reported about 25 bacterial taxa and further emphasized non-occurrence of terrestrial forms.

The microbial communities isolated from the gills of the mullet along with the morphological and biochemical tests are given in Table 2. Five different bacterial strains isolated from the gill surfaces were mainly represented by *Pseudomonas* spp., *Azotobacter* spp., *Proteus vulgaris*, *Streptococcus* spp. and *Planococcus* spp. The observations from gill surfaces revealed that majority of the strains were aerobic, however one strain was found to be facultative anaerobic thus indicating prevalence of aerobic environment in the vicinity of gills. Published literature [10] suggest the dominance of *Pseudomonas* spp. on the gills of freshwater salmonid, however Shewan [11] opined that although the microflora on the gills differ qualitatively among freshwater and marine fish, the density appears to be of same magnitude. Such low density has been attributed to the fact that the bacteria populate only a small portion of available normal gill surfaces. The reasons for variation in population levels in different organs are complex reflecting subtle nutritional and physico-chemical variations within ecological niches. This permits localized response by selected components on the micro-flora. The micro-flora associated with gills are likely to have significant effect on fish as constant movement of water over gills might provide opportunity for contamination and colonization.

The bacterial strains isolated from the intestinal tract of the mullet (Table 3) indicate diverse occurrence of different bacterial strains. Earlier studies [5,12] suggest that microorganisms form an

important dietary component for deposit feeding animals. Further, it has been demonstrated that about 15 – 30 % of organic source in the stomach of mullets has been contributed by microorganisms. Such occurrence of diversified population of bacteria also indicate their possible role in breakdown of plant matter as the gut content of mullets also harbor sizable quantity of plant matter [4]. The present study indicate that eight species were isolated from the intestinal tract of mullet and were represented by *Pseudomonas* spp., *Vibrio parahaemolyticus*, *Vibrio* spp., *Proteus vulgaris*, *Bacillus subtilis*, *Serratia marcescens*, *Enterobacter* spp. and *Streptococcus* spp. Moriarty [5] reported some of these bacterial strains from the intestinal tract of mullet and prawn. The muramic acid in the gut content of mullet was high indicating ingestion of bacterial strains associated with sediments thus forming an important component of detritus based food chain. It was also found that about 80 % of the microbial communities were represented by gram negative whereas only 20 % contribution came from gram positive. It has been well documented that diatoms are an important component of food of mullets, however the bacterial contribution is of equal magnitude wherein the proportions of protozoans attached to sediments is significant [4].

A comparative analysis of the microbial communities in different organs (surface, gills, intestinal tract) showed that high diversity was noticed in the intestinal tract followed by surface and gills (Tables 1-3), and also revealed the dominance of gram negative bacterial strains in the intestinal tract of the mullets. Such occurrence of gram negative forms were earlier reported by Moriarty [5] and stated that the ingestion of bacterial population in the food chain at sediment water interface could be influenced by supply of large quantity of allochthonous organic matter. An important observation made in the present study was non-occurrence of *Azotobacter* spp. in the gut content whereas the same was found to be associated with surface and gills. The importance of enzymes in the intestinal tract of mullet have been highlighted and the microorganisms responsible to display proteolytic activity are *Pseudomonas* spp., *Vibrio parahaemolyticus*, *Vibrio* spp., *Proteus vulgaris*, *Serratia marcescens*, *Streptococcus* spp. and *Enterobacter* spp. and amylolytic activity are *Bacillus subtilus*, *Streptococcus* spp., and *Pseudomonas* spp. On the other hand, the microbial strains such as *Vibrio parahaemolyticus*, *Bacillus subtilis* and *Serratia marcescens* were found only in the intestinal tract of the mullets. The occurrence and abundance of such strains in the gut content emphasize their possible role in the enzymatic breakdown of food particles [13].

For assessment of evaluation of the role of the isolated colonies in the protein, lipid and carbohydrate metabolism, the isolated colonies were subjected to growth in the nutrient agar medium so as to ascertain the production of various enzymes such as proteases, amylases and lipases. The results obtained indicate that the isolated colonies play an important role in influencing the proteolytic and amylolytic activity. Philips & Perumalsamy[13] reported the proteolytic bacterial population from the intestinal tract of the prawn and stated that it was mainly dominated by the *Vibrio spp*. The results obtained on the ability of isolated cultures for the activity of lipases and cellulases, do not suggest an active involvement in the breakdown of cellulose. In contrast, Vaidya *et al.* [14] and Venugopal *et al*. [15] have reported marine bacterial strains those produce cellulases in the different media and emphasized the role of heterotrophic bacteria in degradation and transformation of marine plant biomass.

The present study highlights the diversified nature of microbial population in different organs (surface. gills and intestinal tract). Further, the observations also suggest that the isolated bacterial colonies are not same as that of microbial population from surrounding water. Secondly the microbial communities isolated from intestinal tract indicate that they play an important role in enzymatic breakdown (protease and amylase), thus enhancing assimilation of organic molecules. Such ability of few microbial colonies suggests that there exists an increased scope for application and utilization of theses colonies as pro-biotics in fish culture systems to enhance food assimilation, thus influencing production.

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