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Effect of Fish Visceral Protease Enzyme on Growth and Survival of Fresh Water Fish *Catla catla* and the Diversity of Enzymatic Bacterial Population in Gastro Intestinal (GI) Tract

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A B S T R A C T

Protease enzyme plays an essential role in the growth and survival of all living organisms. Fish processing in India generates over 30,000 tonnes of waste in the form of visceral mass and the enzymes present in this waste needs to be stabilized. The present study focus on the utilization and growth of *Catla catla* fed with fish visceral protease enzyme along with diversity of bacterial population in the gut. Maximum production of 2.42±1.02 was attained in *Catla catla* those fed on Experimental (D3). Temperature, pH and Dissolved Oxygen varied between 27±0.14°C to 31±0.14°C, 7.7±0.14 to 8.2±0.12 and 3.1±0.60 mg/l to 3.4.1±0.76 respectively. The Proximate composition (%/ml) of control and FPH samples was also analyzed. The percentage occurrence of protease positive colonies was registered was 55.36%, lipase positive colonies were registered was 23.21% and amylase positive colonies were registered was 21.43% respectively.

***Key words*:** Fish viscera, Protease, Micro flora, Digestive enzymes, Growth rate

Aquaculture – the forming of aquatic organisms including fish, molluscs, crustaceans and aquatic pant is the fastest growing food production system globally, with an increase in production of animal crops of about 9% per year since 1985 [1]. India is endowed with a rich aquatic biodiversity of over 2319 finfish species, which includes 838 freshwater, 113 brackish water and 1368 marine fishes [2-3]. Since the 1970s, global aquaculture production has increased 10% annually and is now the fastest growing food production sector in many countries. The major requirement of fish culture is the efficient transformation of dietary protein into tissue protein [4]. From a practical point of view, the ideal situation should tend to maximize the use of dietary protein for growth, minimizing the use of proteins for functional protein synthesis, gluconeogenesis, lipogenesis and energy. If adequate protein is not provided in the diet, there is a rapid reduction or cessation of growth and a loss of weight due to withdrawal of protein from less vital tissues to maintain the functions of more vital tissues.

Many aquaculture formulations still have fish meal included at levels in excess of 50%. As a strategy to reduce risk, the identification development and use of alternatives to fishmeal and oil in aquaculture diets remain a high priority. As a result, the volumes of fishmeal and oil is used in aquaculture especially carnivorous species was high rather than the quantity of fish produced, and this practice has raised concern about the long-term sustainability of these industries [5]. Protein digestibility as measured by mink has been shown to be a good indicator for protein digestibility values in fish [6-7] shows significantly reduced values with increased processing temperatures. The digestive tract or the viscera, which constitutes 5 to 8% of the weight, is usually wasted [8]. This material has a nutritional value equivalent to that of whole fish [9] and has been usually converted into silage which is suitable as an easily digestible protein supplement for juvenile fish [10] and as a health supplement for ruminants [11].

The biological diversity of marine and estuarine fish species provides a wide variety of enzymes with unique properties. In recent years, protease from the gut of fishes received much attention [12]. It contributes to the development of highly added applications or products by using the enzyme aided digestion of proteins from different source including from marine animals. Gut microflora plays an important role in the digestive process, growth and disease of the host. It is capable of producing proteolytic, amylolytic, cellulolytic, lypolytic and chitinolytic enzymes which is important for digestion of Proteins, Carbohydrates, Cellulose, Lipids and Chitin above all some pathogen inhibitory compounds [13]. The GI tract of fish is densely populated with microorganisms much higher than those in the aquatic medium, suggesting supportive ecological niches of these

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microorganisms in the tract [14]. It was reported that analysis of digestive enzymes activities in easy and reliable methodology that can be used as indicators of digestive process and nutritional conduction of marine organisms. Therefore, the present work focus on the growth and survival of *Catla catla* using crude and partially purified fish visceral protease of different concentration along with the distribution of enzymatic bacterial population in various part of the GI tract (FG, MG and HG).

MATERIALS AND METHODS

In the present study, fresh water fish *Catla catla* were selected to find out growth, survival and distribution of enzymatic bacterial population.

Biochemical analysis of raw fish viscera and FPH

Biochemical parameters like moisture, ash content, protein and carbohydrate were estimated, AOAC [15].

Preparation of feeds

Visceral wastes of selected marine fish *Sphyraena obtusata* were collected from the local fish market near Tirunelveli. These fish visceral wastes were homogenized with 0.02M Tris- HCl, pH 8.0. The homogenate was centrifuged at 6000 rpm for 15 min and the supernatant referred to as "crude extract". Further the Crude extract was partially purified through Ammonium Sulphate Precipitation, Ultra-filtration and Dialysis.

The feed ingredients were weighed and mixed well in a container by adding sufficient quantity of distilled water and then the ingredients were made into dough. The dough was then placed in a container and boiled in a pressure cooker for 15 minutes. After boiling, the dough was taken out of the container and then vitamins and mineral mixture, cod liver oil, were added to the dough and mixed well. The experimental diet was mixed well with crude and partially purified Fish Visceral Protease whereas; the control diet lacked the crude and partially purified Fish Visceral Protease.

The dough was then allowed to pass through a pelletizer having perforation diameter of 1.5mm in the die. Then the control as well as the experimental diets was dried in a hot air oven at a temperature of 50°C over night. Then the dried pellets were collected and stored in air tight plastic containers for further use.

Experimental setup and feeding

For the present study, four different groups of *C. catla* weighing about 1.75 ± 0.36 to 2.24 ± 0.78 was cultured separately in 100L FRP tanks that consisted of well aerated fresh water. Each tank comprised of 10 fishes and the experiment was carried out for duration of 30 days. During experimentation fishes were fed with respective diets i.e. (Control and experimental diets) two times a day. Group A was fed with prepared pelleted diet with no FVP enzyme and was considered as control group. Group B and Group C was fed with prepared pelleted diet with 5ml and 10 ml of crude FVP enzyme respectively. Similarly, Group D and Group E were fed with prepared pelleted diet with 5ml and 10ml of partially purified FVP enzyme.

Every 10 days of sampling, the fish were taken for wet weight and complete length measurement. Water quality parameters were maintained at an optimum level by providing 50% of water exchange daily. Mortality, external indications of infections and behavioural abnormalities were recorded every day. The tanks were maintained with proper aeration using Boyu Air Pump U 9900 throughout the experiment. The unfed remains were siphoned out collected and finally dried using a Petri dish in a hot air oven at temperature of 50°C. Growth response of experimental organism noted every 10 days of experimentation.

Water quality parameters

The water quality parameters were monitored and maintained at an optimum level during the entire experimental duration. Water samples were collected once in 10 days from respective tanks and they were analyzed. Temperature, pH and DO in culture tanks were measured and recorded.

Isolation, identification and screening of extra cellular enzyme of bacterial flora

To assess the population density and diversity of enzymatic bacterial population in the Gastrointestinal Tract (GI tract) of *C. catla*, the fishes were dissected out in an aseptic condition and then GI tract region such as FG, MG and HG were collected. After a homogenate solution was made by grinding FG, MG and HG with 1 ml of distilled water. Serial dilutions 10^{-2} to 10^{-6} were made and bacterial colony were isolated using Nutrient agar.

Morphologically dissimilar and well isolated colonies were randomly selected after observing the morphology and pigmentation of the colony. The bacterial strains isolated from the fish samples were identified by Bergeys Manual of determinative bacteriology. After identification of different bacterial genera, the isolates were screened for the production of extra cellular protease, extracellular amylase production and extracellular lipase production.

RESULTS AND DISCUSSION

Growth responses of Catla catla fed on control (C) and experimental diets (D1 – D4) during 30 days of experimental duration

It was observed that, there is a maximum production of 2.42±1.02 was attained in *Catla catla* those fed on Experimental (D3). However, the minimum production of 1.1±1.01 was attained on fishes those fed with Control Diet (C). There is an obvious fluctuation in FCR was observed in *Catla catla* fed on different diets. The FCR value varied between 1.43±0.21 and 2.73±0.28 respectively in different diet fed groups. The higher FCE was observed in Experimental Diet D3 (69.54±1.36) whereas lower FCE was observed in Control Diet C (36.544±1.20). Likewise, the higher SGR was observed in Experimental Diet D3 (8.06±1.36) whereas lower SGR was observed in Control Diet C (3.66±1.12).

Water quality parameters of Catla catla fed on different diets during the end of experimentation

The water quality parameters were recorded during the culture of *Catla catla* in various tanks. Specifically, the temperature fluctuated between 27±0.14°C to 31±0.14°C. Similarly, the Dissolved Oxygen content and pH varied between 3.1±0.60 mg/l to 3.4.1±0.76 mg/l and 7.7±0.14 to 8.2±0.12 respectively.

Proximate composition (%/ml) of control and FPH samples

Biochemical constituents of unhydrolyzed raw material and hydrolysate protein prepared through autolysis were analyzed. It was observed that protein content was high in

Control (64.1±0.21) than FPH (62.1±0.51) (Likewise, Carbohydrate content (19.1±0.12) and Ash content (20.2±0.12) was also observed as high in Control than FPH. Similarly, the high moisture content observed was in Control (24.1±0.08) and low moisture content was observed in FPH (12.7±0.06). Likewise, high Lipid content was observed in Control (29.8±0.18) and was observed low in FPH (18.7±0.14). The Two-way ANOVA result revealed that there is significant variation in proximate composition between control and FPH ($P < 0.05$).

Table 1 Amount of feed ingredients used for the preparation of control (C) and experimental diets

Feed ingredients	Diet arrangement				
	Control (C)	Experimental diet (D1)	Experimental diet (D2)	Experimental diet (D3)	Experimental diet (D4)
Fish meal (g)	30.00	30.00	30.00	30.00	30.00
Ground nut oil cake (g)	27.00	27.00	27.00	27.00	27.00
Soya meal (g)	27.00	27.00	27.00	27.00	27.00
Wheat flour (g)	4.30	4.30	4.30	4.30	4.30
Rice bran (g)	4.30	4.30	4.30	4.30	4.30
Tapioca powder (g)	4.40	4.40	4.40	4.40	4.40
Vitamin and mineral premix (g)	2.00	2.00	2.00	2.00	2.00
Cod liver oil (g)	1.0	1.0	1.0	1.0	1.0
Crude FVP (ml)	-	5.00	10.00	-	-
Partially purified FVP (ml)	-	-	-	5.00	10.00

Table 2 Growth responses of *Catla catla* fed on control (C) and experimental diets (D1 – D4) after the 30 days of experimental duration (Mean±SD)

Parameters	Growth responses				
	Control (C)	Experimental diet (D1)	Experimental diet (D2)	Experimental diet (D3)	Experimental diet (D4)
Initial weight (g)	1.8±1.01	2.2±1.12	2.54±1.17	3.07±1.21	2.97±1.22
Final weight (g)	2.9±1.43	3.56±1.42	4.2±1.26	5.49±1.20	4.99±1.30
Production (g)	1.1±1.01	1.36±1.02	1.66±1.06	2.42±1.02	2.02±1.03
Food consumed (g)	3.01±1.11	3.21±1.13	3.24±1.12	3.48±1.11	3.45±1.20
FCR	2.73±0.28	2.36±0.25	1.95±0.20	1.43±0.21	1.70±0.24
AGR (g/body weight/day)	0.036±0.003	0.045±0.001	0.055±0.003	0.080±0.002	0.067±0.001
FCE (%)	36.544±1.20	42.36±1.22	51.23±1.28	69.54±1.36	58.55±1.22
SGR (%)	3.66±1.12	4.53±1.20	5.53±1.31	8.06±1.36	6.73±1.25

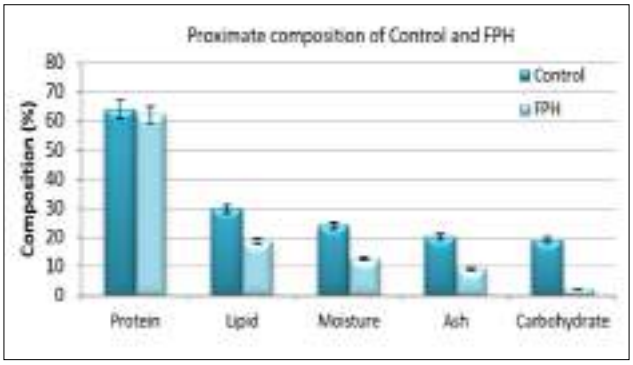

Fig 1 Proximate composition (%/ml) of control and FPH samples

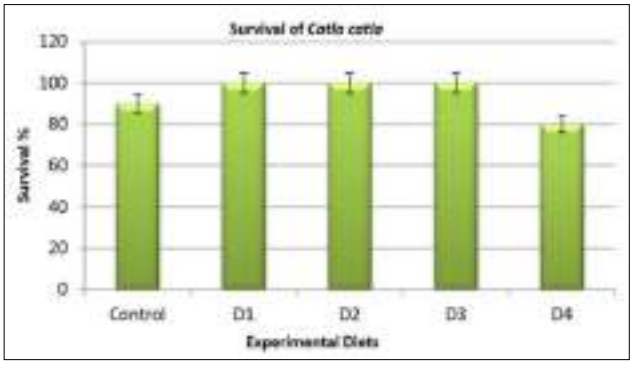

Fig 2 Survival of *Catla catla* fed on different diets during the end of the experimentation

Survival of Catla catla fed on different diets during the end of the experimentation

The survival of *Catla catla* fed on different diets during the end of the experimentation was observed. The survival was high 100% in Experimental Diet (D1), Experimental Diet (D2) and Experimental Diet (D3). Low survival rate of 90% was observed in Control Diet and 80% was observed in Experimental Diet (D4).

Isolation of microbiota from the intestine of Catla catla

Totally 28 morphologically unique bacterial strains were selected from the gut of *Catla catla* for further studies. The colony of these isolates showed varied morphological characters. The bacterial colonies were well grown on nutrient agar media and the morphology of colonies was identified and the single colony was sub cultured. The obtained cultures were named as CS1-CS28 (Catla Strain).

Screening of proteolytic, amylolytic and lipolytic bacteria isolated from gut of C. catla

The isolated bacterial colonies from the gut *C. catla* were qualitatively screened for protease, lipase and amylase production. The zone of clearance surrounding the bacterial growth on skim milk agar plate indicated protease enzyme production, bacterial growth on starch agar plate indicates amylase enzyme production and bacterial growth on spirit blue agar plate indicated lipase enzyme production. Twenty-eight bacterial isolates were examined for the enzyme production. The percentage occurrence of protease positive

colonies were registered was 55.36%, lipase positive colonies were registered was 23.21% and amylase positive colonies were registered was 21.43% respectively.

The fish raw material has an increasing demand recently. The present study correlates with many investigations on proteases activities of several marine species so as to develop an effective diet through the proper awareness of their digestive capacities towards various feed ingredients for intensive farming of these species. In Accordance with [16] due to the addition of dried fish viscera as a fat rich dietary component, a higher level of lipid was estimated in the prepared diet. Likewise, Weight gain was positively correlated with the overall capacity of the digestive enzyme to hydrolyse the diets in *Chinook salmon* [17].

Table 3 Water quality parameters recorded in control tank (C) during experimental duration (Mean±SD)

Parameters	Control (C)			Experimental (D1)			Experimental (D2)			Experimental (D3)			Experimental (D4)		
	10th day	20th day	30th day	10th day	20th day	30th day	10th day	20th day	30th day	10th day	20th day	30th day	10th day	20th day	30th day
Temperature (°C)	28± 0.12	29± 0.13	28± 0.12	27± 0.12	30± 0.13	29± 0.12	29± 0.11	30± 0.12	30± 0.10	25± 0.13	29± 0.14	29± 0.11	29± 0.12	29± 0.12	31± 0.14
pH	8.0± 0.14	8.0± 0.14	8.2± 0.15	7.9± 0.14	8.2± 0.15	7.9± 0.14	7.9± 0.13	8.1± 0.11	8.0± 0.18	7.7± 0.14	8.2± 0.12	8.0± 0.17	7.8± 0.13	7.9± 0.14	7.9± 0.14
DO (mg/l)	3.1± 0.60	3.2± 0.79	3.41± 0.73	4.01±0 .75	3.8± 0.74	4.0± 0.72	4.1± 0.76	3.7± 0.73	3.91± 0.69	3.9± 0.78	3.8± 0.74	3.9± 0.71	3.8± 0.63	3.7± 0.6	3.8± 0.68

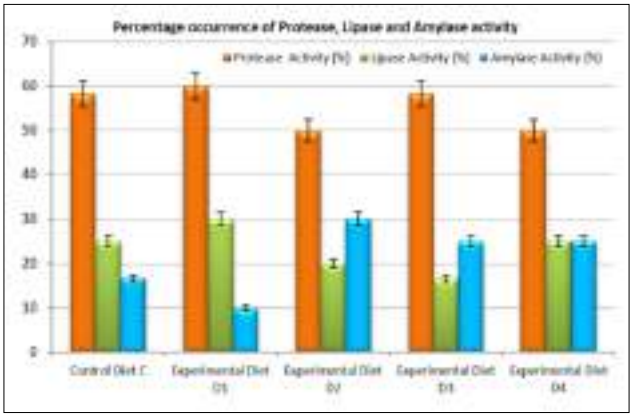

Fig 3 Percentage occurrence of protease, lipase and amylase activity of bacterial strains isolated from the gut of *C. catla* from control diet, experimental diet D1, experimental diet D2, experimental diet D3, experimental diet D4

Table 4 Proximate composition (%/ml) of control and FPH samples (Mean±SD)

Composition (%)	Control	FPH
Protein	64.1±0.21	62.1±0.51
Lipid	29.8±0.13	18.7±0.14
Moisture	24.1±0.08	12.7±0.06
Ash	20.2±0.12	9.27±0.15
Carbohydrate	19.1±0.12	2.1±0.13

Temperature, pH and Dissolved Oxygen are a prevalent factor which affects the food consumption, growth, and production of fishes [18]. The Present results are in line with many observations. In poiklothermic vertebrates including fish, the growth performance and nutrient requirement was affected by the influence of water on metabolic temperature rate and energy expenditure [19-20]. Decline in water temperature creates sluggishness and retards the capacity of digestion [21] and acidic water pH (5.5) limit the growth of the fish and reproduction [22]. The ideal pH for aquaculture in fresh water should be in the range of 6.5 and 7.00 [23]. Dissolved Oxygen has a vital role in the growth, distribution, survival, behavior and overall physiology of aquatic organisms [24]. Decrease of oxygen content directly or indirectly leads to starvation, reduction in growth, fish mortality and poor feeding of fishes.

In the present study, proximate composition of unhydrolyzed raw material and hydrolysate protein prepared through autolysis were analyzed. Autolytic method was commonly used to recover protein from underutilized fish or fish processing wastes [25]. High protein content of control and protein hydrolysate demonstrates its potential use as protein supplements for human nutrition. Decreased lipid content in FPH may be due to the lipid oxidation caused by the exclusion in centrifugation [26-27]. Similarly, carbohydrate level was high in raw sample of catfish *Clarios gariepinus* [28].

Protease activity Lipase activity Amylase activity

Fig 4 Protease, lipase and amylase activity of bacterial strains isolated from gut of *C. catla*

The difference in carbohydrate may be due to the change in temperature. Carbohydrate concentration in *Tilapia zillii* was 4.72% [29]. Likewise, the increased ash content was also reported in the prepared diet, caused by the presence of fish meal as a major ingredient. Ash content in head region of *O. mossambicus* was greater than muscle region [30]. Similarly, the elevated moisture contents in organisms are considered as a benefit, because of its involvement in the stabilization of the organisms during movements. In *P. gigas* the moisture content observed was greater in ventral region when compared with dorsal and lateral line [30].

The digestive enzymes can support nutritional strategies for the diet formulation and feeding of fishes [31]. In *Cyprinus rubrifucus* majority of the isolates associated with the intestinal tract showed Lipolytic activity. In line with the present study various studies have also established that microbial flora in digestive tract of fish exhibited lipolytic, proteolytic, amylolytic and cellulolytic activities [32-33].

CONCLUSION

From the afore mentioned investigation it could be concluded that maximum production of 2.42±1.02 was attained in *Catla catla* those fed on experimental (D3The

percentage occurrence of protease positive colonies was registered was 55.36%, lipase positive colonies were registered was 23.21% and amylase positive colonies were registered was 21.43% respectively.

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