PROJECT TITLE: EFFECT OF REPLACEMENT OF FISH MEAL IN COMMERCIAL AQUAFEEDS BY SOYBEAN ON GROWTH AND METABOLISM OF MOST VALUABLE FRESHWATER CULTIVATED FISH SPECIES.

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INTRODUCTION

The formulation of fish feed is a key to the success in commercial fish culture. A large number of indigenous raw materials mainly poultry by-product meal, blood meal, various oilcakes, cereal by-products, leaf meals etc. are available in the country (Akand et al 1991). These raw materials can be used in developing supplemental feed for rearing and culture of different fish species (Bhadra et al 1997). For the good result of fish production, good quality artificial feed is essential and requires protein level of 35-45% in feed (Degani et al 1989). Dietary protein is used by fish for growth, energy and maintenance (Kaushik and Medale 1994). Protein requirement for maximum growth of any species is a logical step to the development of a cost-effective feed for the fish, and entails determining the minimum amount required to produce maximum growth and not be used for energy (Sang-Min and Tae-Jun 2005). Thus, any reduction in dietary protein level without affecting fish growth can substantially reduce the cost of feed. The need for protein and other nutrients in supplemental diets depends upon the levels supplied by the natural food for a targeted production level. Thus, the incorporation of these nutrients in supplemental diets must increase to meet the requirement of increasing fish biomass (Sumagaysay and Borlongan, 1995). Protein, essential for tissue growth and maintenance, is an expensive component of formulated diets. When insufficient energy is available in a diet from non-protein sources, protein may be catabolised to meet the energy requirements at the cost of nutrient supply (Capuzzo & Lancaster 1979 and Sedgwick 1979). Hence the aim of our study was to study the effect of replacement of fish meal as main protein source with soybean meal alternate protein source in diet of Indian catfish.

The role of artificial feed in intensive fish farming cannot be ignored as nutritional requirements of fish depend upon the feed supplied. The quantity and quality of feed consumed have a pronounced effect on growth rate, efficiency of feed conversion and chemical composition of fish (Hassan *et al.,* 1996; Jena *et al.,* 1998; Erfanullah and Jafri, 1998). In this present systematic study aims to assess the growth and digestibility of Indian major carp *Cirrhinus mrigala* fry with different type of feeds ingredients in

formulated diets. Fish fry were fed formulated pellet incorporated with different nutritional supplements like groundnut oil cake, raw soybean, autoclaved soybean and fish meal at different concentrations.

The presence of microbial flora in fish could play diverse roles some of which might be beneficial to the fish itself. The bacterial composition may change with age, individuals, nutritional status, environment conditions, and the complexity of the fish digestive system (Cahill 1990; Ringø et al. 1995; Al-Harbi and Uddin 2004). Intestinal bacterial flora is also important in nutrition. Kwashiwada et al. 1970 showed that vitamin B12 was produced by microorganisms in the intestine of carp. It is generally believed that bacteria can contribute to the diet of fish (Bowen 1976). The intestinal flora of endothermic animals serves both a digestive function and as a protection barrier against disease. Assuming that the non-pathogenic microflora has a disease-preventive effect, this protection is likely to be mediated by microorganisms that are present in high numbers.

Different studies have been performed on the indigenous microflora of fish in aquaculture. They include the study of the bacterial microflora associated with fish farms (Allen et al., 1983) and the nutritional role of the intestinal flora (Goodrich and Morita, 1977; Sugita et al., 1991). The microbiology of the intestinal tract of marine and freshwater fish has been investigated by different researchers and most of them have aimed to determine the origin of the organism responsible for the spoilage of freshly caught fish. These surveys have demonstrated that the quality and the quantity of bacteria are a reflection of different factors: one of the factor is diet (Austin and Al-Zahrani, 1988; Ringo and Olsen, 1999; Ringo et al., 2006; Heikkinem et al., 2006). As a consequence, the study of the gut microflora is considered important in aquaculture because it reflects both the bacterial composition of the rearing environment (water) and the dietary regimen of ingested food. Intestinal microflora has been considered an important component of the digestive tract in animals including fish. The intestinal microflora must adapt to various conditions of nutrient composition, pH, anaerobiosis, concentration of bile salts and digestive enzymes, the hosts‟ immune system, and the presence of other members of intestinal community. According to Sugita et al., (1988), the development of the gut microflora in *Carassius auratus* has three stages: the transitory (accidental) microflora, which is scarce, does not remain for long in the intestine, and occurs also in the water, food, and on the surface of fish eggs; Most of the

studies on fermentative bacterial activity are referred to herbivorous fish (Luczkovic and Stellwag, 1993; Clements and Choat, 1997; Mountfort et al., 2002), omnivorous (Kihara and Sakata, 2002) and detritivorous teleosts (Kihara and Sakata, 1997) but various studies have also been performed on carnivorous fish (Kihara et al., 1995; Kihara and Sakata, 2001; Mahious et al., 2006; Burr et al., 2010). In this contex, the study of microbiota present in the intestine of fish is of great importance because these bacteria reflect different factors such as the diet, general rearing conditions, aqueous parameters and the general the way of life of the host species. Therefore, studies on the characterization of the dominant microflora are a crucial part in fish research.

OBJECTIVES

1) To study the effect of raw and processed soybean diets on growth performance, digestibility and nutrient retention on different feeding habits of fish species
2) To study the effect of raw and processed soybean diets on post prandial excretory levels of total ammonia and reactive phosphate in holding water.
3) Comparison of digestive enzyme activity in fresh water fish species of different feeding habits
4) Isolation and characterization of digestive enzyme producing bacterial flora form the gut of different feeding habits fish species

OBJECTIVE: 1

To study the effect of raw and processed soybean diets on growth performance, digestibility and nutrient retention on different feeding habits of fish species

METHODOLOGY

For this three fish species were selected i.e. *Carassius auratus,* common goldfish (herbivore), *Cirrihinus mrigala,* mrigal (omnivore), and *Clarias batrachus,* Indian magur (carnivore)

Diet preparation

- Before incorporating in diet soybean is hydrothermically processed (for 15 min at 121°C at 15 lbs) after then eight diets (1-4 raw soybean based, 5-8 processed soybean based) with 40% protein level were formulated by blending raw and processed soybean at four different inclusion levels viz. 65, 130, 195 and 260g kg^{-1} with fish meal.
- A diet with fish meal as the protein source were used as reference diet.
- Ground nut oil cake and rice bran were used as base materials. Wheat flour were added as a binder while and 1% chromic oxide were added as an external digestible marker for digestibility estimations. All diets were supplemented with a mineral premix (MPA).
- After then by using a mechanical pelletizer, 0.5mm thicker pellets were obtained which then dried in oven (60-62°C) before using in feeding trials.

Experiment design

- The fry of freshwater cultivated fish species were obtained from near by fish farm and acclimated in glass aquaria (60×30×30cms) for a minimum period of 10 days prior to the commencement of experiment. The temperature and lighting schedule of laboratory were maintained $25\pm1^{O}C$ and LD 12:12 respectively. The aquaria water were renewed daily with water adjusted to the laboratory temperature ($25^{O}C$).
- Fry (mean body weight 0.55) were randomly distributed @ 15 fish per aquarium with three replicates of each dietary treatment.
- All fish were fed daily twice at 08^{00} h and in afternoon at 4.00 p.m. The feeding rate being 5% BWd^{-1} for the whole duration and the feeding rates were adjusted every fifteen days after bulk weighing each group of fish were exposed to their respective diet for four hour during each ration, thereafter, the uneaten feed were siphoned out, stored and dried separately for calculating the feed conversion ratio (FCR).
- The faecal matters voided by the fish were collected by siphoning separately from each aquarium. The faecal samples were dried in a hot air oven at $60^{O}C$ and subsequently analyzed for digestibility estimations.
- At the termination of experiment, the fish from all the treatments were weighed (length will also be recorded) individually to the nearest gram and processed for subsequent analyses.

Data collection and Analytical techniques

- The feed ingredients, experimental diets, feacal matter samples, fish carcass (Initial and final) were analyzed following the procedure of AOAC (1995).
- Live weight gain (g), growth percent gain, specific growth rate (% d^{-1}), feed conversation ratio (FCR), protein efficacy ratio (PER), gross protein retention (GPR) and gross energy retention (GER) were calculated using standard methods (Steffens 1989).
- Cr_2O_3 levels in the diets as well as in the feacal samples were estimated spectophotometrically following the method of Furukawa and Tsukahare (1996).
- pH and dissolved oxygen were monitored using an automatic analyzer (F-set-3 E. Merck Germany).

- Apparent nutrient digestibility of the diets were calculated according to Cho *et al*. (1982).
- Energy content of the diets and fish were calculated using the average caloric conversion factors of 0.3954, 0.1715 and 0.2364 KJg^{-1} for lipid carbohydrate and protein respectively (Henken *et al*. 1986).

RESULTS AND DISCUSSION

The results showed that goldfish fed on processed soybean diet significantly ($P < 0.01$) increase in Growth (%gain in body weight) and specific growth rate (SGR) and maximum increase in these parameters were observed in fish which fed on 100% processed soybean based diet-8th. Carcass phosphorous levels were significantly ($P < 0.05$) higher in fish fed the fishmeal control diet. It is concluded that fishmeal could be completely replaced by processed full-fat soybean in diet of gold fish and hence have positive effect on growth performance of fish.

***Carassius auratus* (gold fish)**

The results of the growth performance, nutrient retention and food conversion efficiency of goldfish fry is presented in Table 1. The present finding indicated that the total replacement of FM with processed soybean meal significantly ($P<0.01$) increased the growth performance of goldfish. Weight gain (%gain in body weight) of fish in this study was significantly affected by processed soybean meal based diets. FCR values also differ significantly ($P<0.01$) between the control group, fish fed on diets containing raw soybean and fish fed on diets containing processed soybean. Protein efficiency ratio (PER) was noticeably different between treatments and supported the same trend. The fish fed the processed soybean diet displayed superior PER while fish receiving control diet and the different levels of raw soybean exhibited less quality of PER

Table 1. Replacement of fish meal by soybean on growth performance, nutrient retention and food conversion efficiency in common goldfish fry under laboratory conditions (LD 12:12 25±1^{0}C)

Parameters	**Diets (g kg^{-1})**								
	Reference diet*	1[a]	2[a]	3[a]	4[a]	5[b]	6[b]	7[b]	8[b]
Initial live weight (g)	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Final live weight (g)	3.28	3.16	3.11	2.97	2.66	3.43	3.66	3.87	4.57
Live Weight gain (g)	2.68	2.56	2.51	2.37	2.06	2.83	3.06	3.27	3.97
Growth (%gain in body weight)	446.67 ±0.22	426.67 ±0.21	418.33 ±0.14	395.00 ±0.19	343.33 ±0.21	471.67 ±0.11	510.00 ±0.10	545.00 ±0.09	661.67 ±0.05
Specific growth rate (SGR)	2.98 ±0.04	2.84 ±0.02	2.79 ±0.01	2.63 ±0.04	2.29 ±0.04	3.14 ±0.01	3.40 ±0.01	3.63 ±0.03	4.41 ±0.01
Food conversion ratio (FCR)	15.11 ±0.03	15.82 ±0.07	16.14 ±0.09	17.09 ±0.07	19.66 ±0.02	14.31 ±0.08	13.24 ±0.11	12.39 ±0.04	10.20 ±0.02
Protein efficiency ratio (PER)	0.165 ±0.02	0.158 ±0.03	0.155 ±0.05	0.146 ±0.07	0.127 ±0.11	0.175 ±0.02	0.189 ±0.03	0.202 ±0.01	0.245 ±0.01
Survival rate (%)	100	100	100	100	100	100	100	100	100

All the vales are mean ±S.E of mean. * Fish meal, [a] Raw soybean based, [b] Processed soybean based.

***Cirrihinus mrigala* (mrigal)**

The results of the growth performance, nutrient retention and food conversion efficiency of mrigal fry is presented in Table 3. Growth parameters of mrigal fry with different diets clearly showed significant enhancement with 25% autoclaved soybean and 75% fishmeal when compared with other concentrations of seven diets and control. Mrigal fry showed maximum increase in final live weight (3.03g), Growth (%gain in body weight) (461.11±0.12), FCR (9.24±0.38) and SGR (2.77 ±0.002) were observed in 25% autoclaved soybean and 75% fishmeal based diet (Table-2).

Table 2. Effect of replacement of fish meal by soybean on growth performance, nutrient retention and food conversion efficiency in *Cirrhinus mrigala* fry under laboratory conditions (LD 12:12 25±1^{0}C)

Parameters	**Diets (g kg^{-1})**								
	Reference diet*	**1[a]**	**2[a]**	**3[a]**	**4[a]**	**5[b]**	**6[b]**	**7[b]**	**8[b]**
Initial live weight (g)	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54
Final live weight (g)	2.83	2.26	2.09	2.97	2.88	3.03	2.58	2.37	2.36
Growth (%gain in body weight)	424.07 ±0.32	318.52 ±0.42	287.03 ±0.20	264.81 ±0.33	248.15 ±0.43	461.11 ±0.12	377.78 ±0.20	338.89 ±0.22	337.04 ±0.22
Specific growth rate (SGR)	2.54 ±0.001	1.91 ±0.001	1.72 ±0.003	1.59 ±0.002	1.49 ±0.002	2.77 ±0.002	2.04 ±0.002	2.03 ±0.001	2.02 ±0.001
Food conversion ratio (FCR)	9.89 ±0.23	12.38 ±0.27	13.40 ±0.19	14.21 ±0.37	14.89 ±0.32	9.24 ±0.38	10.85 ±0.30	11.85 ±0.40	11.86 ±0.40
Protein efficiency ratio (PER)	0.204 ±0.03	.154 ±0.03	.138 ±0.05	.149 ±0.01	.120 ±0.04	.222 ±0.01	.182 ±0.01	.163 ±0.02	.163 ±0.01

All the vales are mean ±S.E of mean. * Fish meal, [a] Raw soybean base, [b] Processed soybean based.

***Clarias batrachus* (catfish)**

Results of the present finding indicated that the reference diet significantly (P<0.01) increased the growth performance of catfish. Weight gain (%gain in body weight) of fish was significantly high in reference diet. FCR values also recorded in accordingly and had

significantly (P<0.01) values as compared to other diets. Protein efficiency ratio (PER) was noticeably different between treatments and supported the same trend. The fish fed the processed soybean diet displayed inferior PER except diet-1 while fish receiving control diet exhibited high quality of PER. Hence no significant difference was observed between reference diet and diet-1.

Table 3. Effect of processed diets on growth performance and nutrient retention in catfish fry under laboratory conditions (LD 12:12 25±1^{0}C)

Parameters	**Diets (g kg^{-1})**								
	Reference diet*	1[a]	2[a]	3[a]	4[a]	5[b]	6[b]	7[b]	8[b]
Initial live weight (g)	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57
Weight after 30 days	1.76	1.23	1.26	1.20	1.10	1.98	1.67	1.58	1.48
Weight after 60 days	2.84	2.02	2.04	2.00	1.98	2.81	2.34	2.24	2.10
Final live weight (g)	5.82	3.56	3.21	2.86	2.56	5.43	4.68	4.27	4.07
Live Weight gain (g)	5.25	2.99	2.64	2.29	1.99	4.86	4.11	3.70	3.50
Growth (%gain in body weight)	921.05 ±0.22	524.56 ±0.31	463.15 ±0.24	401.75 ±0.36	349.12 ±0.37	852.63 ±0.18	721.05 ±0.16	649.12 ±0.25	614.03 ±0.17
Specific growth rate (%SGR)/day	1.332 ±0.06	0.785 ±0.08	0.671 ±0.05	0.543 ±0.05	0.420 ±0.04	1.255 ±0.07	1.09 ±0.03	0.988 ±0.07	0.931 ±0.05
Protein efficiency ratio (PER)	0.131 ±0.03	0.089 ±0.05	0.080 ±0.07	0.071 ±0.08	0.064 ±0.12	0.135 ±0.05	0.117 ±0.06	0.107 ±0.07	0.102 ±0.08
Survival rate (%)	86.67	86.67	86.67	80.00	66.67	86.67	86.67	86.67	86.67

All the vales are mean ±S.E of mean. * Fish meal, [a] Raw soybean based, [b] Processed soybean based.

The effect of diet on fish growth depends on several factors including fish species, developmental stages, environmental conditions and nutrient and energy levels in feeds. Ruohonen et al. (1998) found that at least three meals per day were required for maximum

growth of 1-year-old rainbow trout and the number of feeding depends on the nutrient density of feeds. Two feedings per day to apparent satiation were recommended for the maximal growth of yellowtail flounder during the grow-out period (Dwyer et al. 2002). Lee and Pham (2010) reported that the weight gain of juvenile olive flounder was influenced by the feed type and feeding frequency.

In our study FCR values also differ significantly ($P<0.01$) between the control group gold fish, gold fish fed on diets containing raw soybean and fish fed on diets containing processed soybean. Protein efficiency ratio (PER) was noticeably different between treatments and supported the same trend. The fish fed the processed soybean diet displayed superior PER while fish receiving control diet and the different levels of raw soybean exhibited less quality of PER. Hence there are various other benefits of using autoclaved soybean for fish aquaculture including that of carp such as reduction in culture cost of *Cyprinus carpio* (Ghosh et al. 2003) and Indian major carps (Swain et al. 1996). In present findings decrease in growth parameters were observed with the increasing inclusion level of raw soybean in the diet. Our results are in agree with, Mazid et al. (1994); Wilson & Poe (1985); Viola et al. (1983); Sadiku & Jauncey (2002); Garg et al. (2002) where Poor growth performance in tilapia, carp, and mrigal fed diets containing soybean has been attributed to the ANFs present in raw soybean.

Certain anti-nutritional factors (ANF's) are also known to specifically interfere with the digestive enzymes in the gastro-intestinal tract. Furthermore, in relation to the present study a decrease in growth rate was observed in all fish fed raw soybean based diets when compared with the processed soybean based diets and fishmeal reference diet. Investigations of digestive enzyme activities constitute an essential aspect of understanding the physiology of the digestive tract and the nutritional requirements of specific stages of development (Le Moullac et al. 1997). Present study, in which we observed a high amount of digestive enzyme activity (protease, amylase, cellulase and Lipase) in the intestine (Table-) and high crude protein and crude fat (Table-5) in fish fed processed soybean based diets. Protease inhibitors are common anti-nutrient substances in many plant derived nutritional staff of potential value, especially the legumes (Norton, 1991). Also protease inhibitors particularly in oil seeds are known to decrease the growth performance in fish (Liener 1994; Sriket et al. 2011). However, Kuz`mina (1990) observed a high proteolytic potential in non-carnivorous fish. This may be understood; on the basis that plant proteins are more difficult to digest by fish in raw form than in processed form and of course to animal protein and fish meal. The enzyme systems in *C. mrigala* like those of cyprinids that have long guts, are adapted to digest and

absorb nutrients from plant feedstuffs (Bairagi et al. 2002; Garg et al. 2002; Rani 2014). These results demonstrate the suitability of hydrothermically processed full-fat soybean as a dietary protein source for common goldfish when growth, digestibility, nutrient retention and excretion of metabolites are taken into consideration.

There are various other benefits of using autoclaved soybean for fish aquaculture including that of carp such as reduction in culture cost of *Cyprinus carpio* (Ghosh *et al.*, 2003) and Indian major carps (Swain *et al*, 1996). Decrease in growth parameters were observed with the increasing inclusion level of raw soybean in the diet. Our results are in agree with, Mazid *et al.,* (1994); Wilson and Poe (1985); Viola *et al.,* (1983); Sadiku and Jauncey (2002); Garg *et al.,* (2002) where Poor growth performance in tilapia, carp, and mrigal fed diets containing soybean has been attributed to the ANFs present in raw soybean. Feeding mrigal with raw soybean diets resulted in significantly lower carcass protein, fat, and energy, and a higher percentage of moisture. Increased digestive enzyme activity (protease, amylase, and cellulase) also support high digestibility and nutrient retention in fish fed supplemented processed soybean diets as compared with the other diets (Table-). The enzyme systems in *C. mrigala* like those of cyprinids that have long guts, are adapted to digest and absorb nutrients from plant feedstuffs (Bairagi *et al.,* 2002; Garg *et al.,* 2002). In the present studies excretion of wastes (N-NH4 and o-PO4) was significantly ($P < 0.05$) lower in aquaria with fish fed diets containing processed soybean as the protein source. Excretion of metabolites was reduced with each increase in the inclusion level of processed soybean in the diet (Kalla and Garg, 2003; Garg *et al.,* 2002; Singh *et al.,* 2003, 2004) also reported a reduction in N-NH4+ and o-PO4 − levels with the use of processed soybean in fish diets. Since excretory rates of metabolites depend not only on the fish species, but also on the size, temperature, salinity, and other experimental conditions (Porter *et al.,* 1987; Ballestrazzi *et al.,* 1994, 1998), absolute values cannot be compared between different species; however, trends in excretion/production of metabolites and relative magnitude can be compared. These results demonstrate the suitability of hydrothermically processed full-fat soybean as a dietary protein source for *C. mrigala* when growth, digestibility, nutrient retention and excretion of metabolites are taken into consideration.

In the present studies, significantly ($P<0.05$) higher growth and digestibility was observed in *Clarias batrachus* fed on diets containing Hydrothermically Processed Soybean which indicate that partial replacement of Fish Meal (FM) was possible with the incorporation of processed full fat soybean without compromising the growth performance, digestibility and nutrient retention in *Clarias batrachus.* Many authors have reported depression in growth in

fish fed on soybean containing diets. The poor growth obtained in tilapia on replacing FM by raw soybean (Mazid et al 1994) was attributed to the presence of ANFs present in the untreated soybean. Raw legume seeds contain several ANFs (Liener 1980), which may be destroyed by thermal treatment (Garg et al 2002). Huisman and Van der Poel (1991) concluded that trypsin inhibitors can be eliminated by atmospheric steaming (102^0C) for long processing times. However, processing time which exceeds 40 minutes do not further increase apparent digestibility for nitrogen (Garg et al 2002). The apparent protein digestibility (APD) and energy retention increased with increase in the inclusion levels of plant proteins in the diets. These results are similar to those observed on a cyprinid *Labeo rohita* for soybean (Hossain et al 1997). Studies have further revealed that APD had no effect on protein and energy retention of the fish (Bureau et al 2000). In general, the pattern of APD corresponds to growth trends of fish fed on different diets incorporating plant origin proteins.The high protein digestibility of plant origin feeds in comparison to FM based feeds may be related to the carnivores feeding habits of *Clarias batrachus*. These findings strengthen the view that the enzyme system in *Clarias batrachus* are better equipped to digest and assimilate nutrients from plant origin feeds. Significantly ($P<0.05$) higher growth was observed in fish fed on diet-6. The results are in slightly contradictly with Webster et al (1995), who have succeeded in achieving complete replacement of FM by the use of Hydrothermically Processed Soybean in the catfish, *Ictalurus furcatus* with supplement of methionine (Robinson and Menghe 2007) in catfishes. Supplement of MPA was also found to enhance the growth of eels. Studies in which SBM was successfully used as partial replacement of FM were reported for blue catfish, *Ictalurus furcatus* (Webster et al 1995) these results are in agreements of our results.

OBJECTIVE: 2

To study the effect of raw and processed soybean diets on post prandial excretory levels of total ammonia and reactive phosphate in holding water.

METHODOLOGY

$N\text{-}NH_4^+$ and $o\text{-}PO_4$ excretion in holding water:

$N\text{-}NH_4^+$ and $o\text{-}PO_4$ were monitored at the end of experiment (after 90 days for 24 h at interval of 2 h)

Total ammonia excretion (mg kg^{-1} BW d^{-l})

Total ammonia excretion was calculated by using the following formula:

$$\text{Total ammonia excretion} = \frac{\text{NH4-N (mg } 1^{-1}\text{) in aquarium water}}{\text{Fish body weight (kg} \} \text{ per L of water}}$$

Reactive phosphate (mg kg^{-1} BW d^{-l})

Reactive phosphate excretion was calculated by using the following formula:

$$\text{Reactive phosphate excretion} = \frac{\text{O-PO}_4\text{ (mg } 1^{-1}\text{) in aquarium water}}{\text{Fish body weight (kg) per L of water}}$$

RESULTS AND DISCUSSION

Gold fish

Significantly (P < 0.01) lower levels of total ammonia excretion and reactive phosphate productions (mg kg−1 BW d−1) were recorded where the fish fed processed full-fat soybean compared to fish fed raw soybean diets as well as control diet.

Table 4. Replacement of fish meal by soybean on Ammonia and reactive phosphate production in common goldfish fry under laboratory conditions (LD 12:12 25±1^{0}C)

Parameters	Diets (g kg^{-1})								
	Reference diet*	1[a]	2[a]	3[a]	4[a]	5[b]	6[b]	7[b]	8[b]
Total ammonia (mg kg^{-1} b w d^{-1})	3097.40 ±45.05	3058.43 ±42.10	3064.85 ±24.07	3079.71 ±25.92	3094.99 ±52.80	2200.02 ±22.45	1903.13 ±26.20	1511.60 ±30.10	1242.05 ±19.13
Reactive phosphate production (mg kg^{-1} b w d^{-1})	2801.45 ±45.10	2616.74 ±20.20	2850.00 ±43.63	2899.99 ±72.22	2999.83 ±45.02	1697.45 ±28.35	1101.07 ±16.35	1044.00 ±21.00	899.12 ±16.11

All the vales are mean ±S.E of mean. * Fish meal, [a] Raw soybean base, [b] Processed soybean based

C. mrigala

Significantly (P < 0.05) lower levels of total ammonia excretion and reactive phosphate productions (mg kg−1 BW d−1) were recorded where the fish were fed processed full-fat soybean diets compared with the fish fed raw soybean diets.

Table 5. Effect of replacement of fish meal by soybean on Ammomia and reactive phosphate production in *Cirrhinus mrigala* fry under laboratory conditions (LD 12:12 25±1^{0}C)

Parameters	Diets (g kg^{-1})								
	Reference diet*	1[a]	2[a]	3[a]	4[a]	5[b]	6[b]	7[b]	8[b]
Total ammonia (mg kg^{-1} b w d^{-1})	2608.45 ±79.06	2078.65 ±53.10	2169.55 ±27.67	2379.88 ±66.92	2544.93 ±57.80	1267.22 ±27.87	1883.93 ±86.40	1591.63 ±55.19	1394.65 ±69.04
Reactive phosphate production (mg kg^{-1} b w d^{-1})	2500.90 ±55.28	1656.87 ±35.76	1850.30 ±47.68	1999.90 ±35.23	2056.23 ±43.07	899.98 ±58.35	1000.97 ±28.05	1134.04 ±42.02	1200.01 ±66.25

All the vales are mean ±S.E of mean. * Fish meal, [a] Raw soybean base, [b] Processed soybean based

Clarias batrachus

Postprandial excretory levels of NH_3-N and reactive P were found high in the treated waters for the fish fed on experimental diets containing FM as the protein source. The excretion decreased on increasing the inclusion levels of HPS. Peak values in NH_3-N excretion occurred after 6-8h and that of reactive P occurred after

8h of feed distribution. The peak time of excretion of NH_3-N and reactive P for the groups of fish fed on experimental diets containing HPS at varying inclusion levels was slightly earlier than the groups of fish fed on FM as the only source of protein.

Table 6. Total feed given and Effect of processed diets on food conversion ratio (FCR) in catfish fry under laboratory conditions (LD 12:12 25±1^{0}C)

Days	**Feed given (g)**								
	Referene diet*	1[a]	2[a]	3[a]	4[a]	5[b]	6[b]	7[b]	8[b]
1-30	25.6	25.6	25.6	25.6	25.6	25.6	25.6	25.6	25.6
31-60	68.64	47.96	49.14	43.20	36.30	77.22	65.12	61.62	57.72
61-90	110.76	78.78	79.56	72.0	65.34	109.60	91.26	87.36	81.90
Parameters									
Unconsumed food (g)	50.00	54.56	54.98	57.23	58.34	75.89	62.23	64.10	70.87
FCR	2.04	2.11	2.38	2.44	2.45	1.93	1.97	1.99	2.07

* Fish meal, [a] Raw soybean based, [b] Processed soybean based.

Table 7. Effect of processed diets on Ammonia and reactive phosphate production in holding water in catfish fry under laboratory conditions (LD 12:12 25±1^{0}C)

Parameters	**Diets (g kg^{-1})**								
	Reference diet*	1[a]	2[a]	3[a]	4[a]	5[b]	6[b]	7[b]	8[b]
Total ammonia (mg kg^{-1} b w d^{-1})	2207.40 ±45.86	3018.48 ±34.76	3024.35 ±34.85	3029.71 ±28.72	3099.09 ±42.86	2209.02 ±24.48	2013.22 ±46.23	2001.70 ±24.23	2000.06 ±18.93
Reactive phosphate production (mg kg^{-1} b w d^{-1})	1802.95 ±65.10	2717.74 ±44.60	2860.80 ±45.63	2900.00 ±32.24	2909.89 ±35.78	1760.45 ±26.35	1751.08 ±46.0	1724.00 ±41.07	1700.12 ±26.01

All the vales are mean ±S.E of mean. * Fish meal, [a] Raw soybean base, [b] Processed soybean based

Excretion of metabolites was reduced with each increase in the inclusion level of processed soybean in the diet (Kalla and Garg, 2003; Garg et al. 2002; Singh et al. 2003, 2004) also reported a reduction in N-NH_4+ and o-PO_4 − levels with the use of processed soybean in fish

diets. Since excretory rates of metabolites depend not only on the fish species, but also on the size, temperature, salinity, and other experimental conditions (Porter et al. 1987; Ballestrazzi et al. 1994, 1998), absolute values cannot be compared between different species; however, trends in excretion/production of metabolites and relative magnitude can be compared.

In the present studies on *C. mrigala* excretion of wastes (N-NH4 and o-PO4) was significantly ($P < 0.05$) lower in aquaria with fish fed diets containing processed soybean as the protein source. Present results are agree with Kalla and Garg, 2003; Garg *et al.,* 2002; Singh *et al.,* 2003, 2004 as they also recorded reduced Excretion of metabolites with the use of processed soybean in fish diets. These results demonstrate the suitability of hydrothermically processed full-fat soybean as a dietary protein source for *C. mrigala* when growth, digestibility, nutrient retention and excretion of metabolites are taken into consideration.

Fishes excrete nitrogen mainly as ammonia which is found to be influenced by dietary protein quality and quantity (Kaushik and Cowey 1991). The reduction in NH_3-N and reactive P excretion was found to be negatively correlated with the growth and digestibility parameters in *Clarias batrachus* and a positive correlation was observed with FCR values. According to Viola and Lahav (1993) calculated amounts of excreted (not retained) nitrogen per Kg gain was reduced by 20% with the lysine supplemented feeds, as compared too the 30% protein feed. Concomitantly, calculated phosphorous excretion per Kg gain was also decreased approximately by 100%. The decrease in NH_3-N and reactive P excretion in the treated water with the use of proteins of plant origin in feed has important implications on the management of highly intensive farming system. Therefore, more studies are warranted in this area.

OBJECTIVE :3

Comparison of digestive enzyme activity in fresh water fish species of different feeding habits

METHODOLOGY

Digestive intestinal enzyme activity

Fishes were killed for the assays after 48 h without food.

Before the preparation of extracts, the viscerosomatic index (VSI = (visceral weight X 100/weight of fish) was calculated.

Preparation of extracts

Protease, amylase and cellulase activities were determined in whole digestive tract of fish. After extraction, tissues were homogenized in 5 volumes (v/w) of ice-cold distilled water. Homogenate was centrifuged at 10,000 rpm for 1h, at 4°C, and the supernatant was removed for further analysis.

Enzyme assays

Estimation of protease enzymes activity

Reagents

1. 1% Bovine Serum Albumin (BSA)
2. 0.1 M phosphate buffer pH 7.6
3. $CaCl_2$ solution
4. 5% TCA

Proteolytic enzyme activity was measured using Bovine Serum Albumin (BSA) as 1% substrate following the methods of Kunitz (1947). The reaction mixture contained 1ml of substrate solution, 1 ml of 0.1 M phosphate buffer (pH 7.6), 1 ml of $CaCI_2$ and 1 ml of crude enzyme extract. The digestion was continued for one hour of incubation at 37°C. It was stopped with 3 ml of 5% TCA solution. After 10 minutes the precipitates were removed by centrifugation. One portion of the supernatant was tested for proteins left digested with 5 ml of Lowry's reagent. The protein was determined by methods of Lowry et al., 1951. Three separate blanks namely (a) containing buffer only, (b) buffer plus substrate and (c) buffer plus enzyme were used. The instrument was set at zero with blanks (a). Enzyme activity in the assay mixture was obtained by measuring absorbance at 660 nm and subtracting the combined absorbance of blank (b) and (c) from it. The equivalent amount of tyrosine released was calculated from the standard curve of tyrosine. One unit of enzyme activity represents the amount of enzyme activity represents the amount of enzyme required to liberate one µg of tyrosine min-I under assay conditions and expressed ml^{-l} of enzyme. Proteins were estimated by Lowry's et al. (1951) given below:

Reagents

1. 2 per cent (w/v) $Na_2 CO_3$ in 0.1 N NaOH.
2. Per cent (w/v) aqueous solution of sodium potassium tartarate.
3. One per cent aqueous solution of $CuSO_4.5H_2O$
4. Reagents 2 and 3 mixed equal volume and 1 ml of the mixture was added to 50 ml of reagent 1.
5. 1 N Folin's reagent.

Procedure

To 1.0 ml of diluted protein solution, 5 ml of reagent 4 was added and mixed thoroughly. It was allowed to stand for 10 min at room temperature. Then 0.5 ml of reagent 5 was added and mixed immediately. After 30 minutes, blue colour was developed and the optical density was measured at 660 nm using spectrophotometer. A blank was also run using 1:0 ml distilled water in place of protein solution. The amount of protein was calculated from the standard Curve prepared for (20-200 µg) of Bovine Serum Albumin as standard protein.

Estimation of intestinal amylase enzyme activity

Reagents

1. Starch solution (1%)
2. NaCl solution (1%)
3. 3, 5 Dinitrosalicyclic reagents
4. 0.1 Phosphate buffer (pH 7.0)

Intestinal amylase activity was measured using starch solution as 1% substrate following the methods of Bernfield (1955). The reaction mixture contained 1 ml of substrate solution, 1 ml of 0.1M-phosphate buffer (pH 7.0), 1 ml (1%) NaCl and 1 ml of crude enzyme extract. The digestion was continued for one hour of incubation at 37^0C.It was stopped with 0.5ml of 3, 5-dinitrosalicyclic acid. Enzyme activity in the assay mixture. Was obtained by measuring absorbance at 540nm and deducing the value from standard curve prepared by using maltose monohydrate. Soluble protein from the enzyme extract was determined by Lowery's methods. One unit of enzyme activity represents the amount of enzyme activity represents the amount of enzyme required to liberate one µg of maltose min under assay conditions and expressed ml of enzyme.

Estimation of intestinal cellulase enzyme activity

Reagents

1. Microcrystalline cellulose (1%)
2. 3, 5-dinitrosalicyclic reagent
3. 0.1 M Phosphate buffer (pH 7.0)

4. Standard Glucose solution

Intestinal cellulose enzyme activity was measured using microcrystalline cellulose as 1% substrate. The reaction mixture contained 1ml of substrate solution, 1ml of 0.1M phosphate buffer and 1ml of crude enzyme extract. The digestion was continued for one hour of incubation at 37^{O}C. It was stopped with 0.5 ml of 3, 5-dinitrosalicyclic acid. Enzyme activity in assay mixture was obtained by measuring absorbance at 540 nm and deducing the value from standard curve prepared by using glucose soluble protein from the enzyme extract was determined by Lowery's method.

One unit of enzyme activity represents the amount of enzyme activity represents the amount of enzyme required to liberate one µg of glucose min^{-1} under assay conditions and expressed ml^{-1} of enzyme.

RESULTS AND DISCUSSION

Gold fish

Investigations of digestive enzyme activities constitute an essential aspect of understanding the physiology of the digestive tract and the nutritional requirements of specific stages of development (Le Moullac et al. 1997). Present study, in which we observed a high amount of digestive enzyme activity (protease, amylase, cellulase and Lipase) in the intestine (Table-8) in fish fed processed soybean based diets. Protease inhibitors are common anti-nutrient substances in many plant derived nutritional staff of potential value, especially the legumes (Norton, 1991). Also protease inhibitors particularly in oil seeds are known to decrease the growth performance in fish (Liener 1994; Sriket et al. 2011). However, Kuz`mina (1990) observed a high proteolytic potential in non-carnivorous fish. This may be understood; on the basis that plant proteins are more difficult to digest by fish in raw form than in processed form and of course to animal protein and fish meal. The enzyme systems in *C. mrigala* like those of cyprinids that have long guts, are adapted to digest and absorb nutrients from plant feedstuffs (Bairagi et al. 2002; Garg et al. 2002; Rani 2014).

Table 8. Replacement of fish meal by soybean on digestive enzyme activities and viscero-somatic index in common goldfish fry under laboratory conditions (LD 12:12 25±10C)

Parameters	**Diets (g kg^{-1})**									
	Initial	Reference diet	1	2	3	4	5	6	7	8
Specific protease activity [A]	0.57 ±0.14	1.49 ±0.12	1.30 ±0.11	1.21 ±0.14	1.19 ±0.12	1.07 ±0.10	1.56 ±0.13	1.68 ±0.14	1.79 ±0.12	2.10 ±0.11
Specific amylase activity[B]	0.32 ±0.04	0.47 ±0.04	0.39 ±0.02	0.35 ±0.03	0.30 ±0.04	0.24 ±0.02	0.49 ±0.01	0.59 ±0.03	0.68 ±0.02	0.80 ±0.01
Specific cellulase activity[C]	0.90 ±0.12	0.80 ±0.14	0.74 ±0.13	0.69 ±0.12	0.60 ±0.14	0.52 ±0.12	1.49 ±0.13	1.62 ±0.04	1.75 ±0.06	2.21 ±0.05
Specific Lipase activity[D]	0.20 ±0.21	0.71 ±0.12	0.60 ±0.20	0.52 ±0.11	0.50 ±0.09	0.34 ±0.13	0.65 ±0.09	0.68 ±0.06	0.70 ±0.03	0.75 ±0.01
Viscero-somatic index (VSI %)	0.16 ±0.02	0.41 ±0.04	0.32 ±0.11	0.30 ±0.08	0.25 ±0.09	0.20 ±0.11	0.57 ±0.02	0.59 ±0.04	0.63 ±0.01	0.72 ±0.03

Values are mean ±SE of mean. [A]Mg of tyrosine mg−1 of protein h−1. [B] Mg of maltose mg−1 of protein h−1 .[C]Mg of glycogen mg−1 of protein h−1, [D]Mg/mg of protein/h

Mrigal

Increased digestive enzyme activity (protease, amylase, and cellulase) also support high digestibility and nutrient retention in fish fed supplemented processed soybean diets as compared with the other

diets (Table-9). The enzyme systems in *C. mrigala* like those of cyprinids that have long guts, are adapted to digest and absorb nutrients from plant feedstuffs (Bairagi *et al.*, 2002; Garg *et al.*, 2002).

Table 9 Effect of replacement of fish meal by soybean on digestive enzyme activities and viscero-somatic index in *Cirrhinus mrigala* fry under laboratory conditions (LD 12:12 25±10C)

Parameters	Diets (g kg^{-1})									
	Initial	Reference diet	1	2	3	4	5	6	7	8
Specific protease activity [A]	0.57 ±0.17	1.49 ±0.11	1.29 ±0.15	1.24 ±0.16	1.03 ±0.19	1.03 ±0.12	1.56 ±0.02	1.47 ±0.13	1.43 ±0.18	1.40 ±0.19
Specific amylase activity[B]	0.11 ±0.03	0.32 ±0.02	0.19 ±0.00	0.19 ±0.03	0.14 ±0.01	0.14 ±0.01	0.47 ±0.00	0.34 ±0.02	0.30 ±0.01	0.27 ±0.01
Specific cellulase activity[C]	0.19 ±0.34	0.54 ±0.12	0.45 ±0.12	0.35 ±0.22	0.32 ±0.23	0.32 ±0.17	0.67 ±0.02	0.59 ±0.01	0.52 ±0.12	0.50 ±0.11
Viscero somatic index (VSI %)	0.11 ±0.04	0.39 ±0.14	.25 ±0.15	0.19 ±0.14	0.19 ±0.14	0.18 ±0.14	0.57 ±0.12	0.40 ±0.14	0.32 ±0.14	0.32 ±0.10

Values are mean ±SE of mean. [A]Mg of tyrosine mg−1 of protein h−1. [B] Mg of maltose mg−1 of protein h−1 .[C]Mg of glycogen mg−1 of protein h−1.

Catfish

Increased digestive enzyme activity (protease, amylase, cellulose and lipase) were observed in catfish fry. Results also support that increase in growth correlated to digestive enzyme activities. (Table-10).

Table 10. Replacement of fish meal by soybean on digestive enzyme activities and viscero-somatic index in common goldfish fry under laboratory conditions (LD 12:12 25±10C)

Parameters	**Diets (g kg^{-1})**									
	Initial	Reference diet	1	2	3	4	5	6	7	8
Specific protease activity [A]	0.57 ±0.13	1.69 ±0.16	1.28 ±0.19	1.27 ±0.14	1.09 ±0.17	1.07 ±0.10	1.46 ±0.12	1.58 ±0.14	1.69 ±0.14	1.70 ±0.12
Specific amylase activity[B]	0.30 ±0.03	0.37 ±0.04	0.34 ±0.02	0.35 ±0.04	0.31 ±0.05	0.33 ±0.02	0.39 ±0.08	0.39 ±0.03	0.48 ±0.02	0.60 ±0.04
Specific cellulase activity[C]	0.30 ±0.22	0.41 ±0.24	0.44 ±0.13	0.54 ±0.12	0.59 ±0.04	0.52 ±0.22	0.60 ±0.23	0.62 ±0.04	0.63 ±0.04	0.63 ±0.04
Specific Lipase activity[D]	0.50 ±0.11	1.71 ±0.32	0.70 ±0.20	0.62 ±0.11	0.55 ±0.09	0.44 ±0.23	0.78 ±0.09	0.78 ±0.06	1.09 ±0.09	1.06 ±0.09
Viscero-somatic index (VSI %)	0.16 ±0.04	0.51 ±0.06	0.42 ±0.15	0.40 ±0.07	0.42 ±0.08	0.38 ±0.13	0.55 ±0.03	0.59 ±0.04	0.62 ±0.06	0.65 ±0.04

Values are mean ±SE of mean. [A]Mg of tyrosine mg−1 of protein h−1. [B] Mg of maltose mg−1 of protein h−1 .[C]Mg of glycogen mg−1 of protein h−1, [D]Mg/mg of protein/h

OBJECTIVE :4

Isolation and characterization of digestive enzyme producing bacterial flora form the gut of different feeding habits fish species

METHODOLOGY

Microbial culture: Microbial culture of the intestinal mucosa collected from the test fish was carried out for bacterial isolation. For this purpose, the homogenate of the intestinal mucosa of each of the test fish was used after five serial 1:10 dilutions (Beveridge et al. 1991). Samples (0.1 ml) were taken from each dilution and poured aseptically within a laminar flow on sterilized Tryptone Soya Agar (TSA) plates, in duplicate. These culture plates were incubated at 34 °C for 24 h. They were then examined for the development of bacterial colonies. The well-separated colonies with apparently different morphological appearance were streaked separately on TSA plates to obtain pure cultures. Single, isolated colonies from the streaked plates were transferred to TSA slants.

To isolate and enumerate protease, amylase, cellulase and lipase producing bacteria, diluted gut homogenate was poured on peptone-gelatin-agar, starch-agar, carboxymethylcellulose(CMC)-agar, and tributyrin-agar media containing plates, respectively. These culture plates were incubated at 34 °C for 24 h. It was assumed that the microflora, which had formed colonies on the peptone-gelatin-agar, had proteolytic activity. Similarly, it was assumed that the microflora grown on starch plate, CMC plate and tributyrin plate had amylolytic cellulolytic and lipolytic activities respectively. By multiplying the number of colonies formed on each plate by the reciprocal of dilution, colony numbers per unit sample volume of gut homogenate were determined (Rahmatullah and Beveridge 1993).

Screening of isolates for extra-cellular qualitative enzyme production: Isolates were screened for the production of extra-cellular protease, amylase, cellulase and lipase. For extra-cellular protease production, the isolates were streaked on peptone-gelatin enriched nutrient agar (4% gelatin) plates and incubated at 32 °C for 15 h. The appearance of a clear zone around the colony after flooding the plate with 15% HgCl2 indicated the presence of proteolytic activity (Jacob and Gerstein 1960). For screening of amylase producing strains, isolates were streaked on starch (1%) supplemented nutrient agar plates and incubated at 32 °C for 48 h. The culture plates were then flooded with 1% Lugol's iodine solution (Jacob and Gerstein 1960) to identify amylase activity. For screening of cellulase producers, isolates were grown in carboxymethlycellulose (1%) nutrient broth and incubated at 32 °C for 72 h. The amount of reducing sugar produced per microgram protein in the culture broth was determined using dinitrosalisylic acid reagent (Sadasivam and Manickam 1996).Lipase

producers showed a clear zone surrounding their colony in tributyrin plates (1%) (Sangiliyandi and Gunasekeran 1996).

Quantitative enzyme assay: Liquid media were used for quantitative assay of enzyme production from different strains. For protease, amylase and cellulase, selective media (without agar) were used. For lipase production the medium contained (mg/ml): Olive oil, 1% (v/v); Sucrose, 5; $MnSO_4.H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.01; Na_2HPO_4, 3; KH_2PO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $NaNO_3$, 5. The culture flasks were incubated for 48 h at 32 °C. The contents were centrifuged (10,000 × g, 10 min, 4 °C) and the cell-free supernatant was used for enzyme assay. The protein content of the enzyme extract was estimated spectrophotometrically at 660 nm according to Lowry et al. (1951).

Protease assay: Protease activity was detected by caseinase assay method (Walter 1984).

Amylase assay: Amylase was assayed by the dinitrosalicylic acid method based on the estimation of reducing sugars at 560 nm using maltose as the standard (Bernfield 1955).

Cellulase assay: The production of reducing sugars due to cellulolytic activity was measured following dinitrosalicylic acid method at 540 nm using glucose as the standard (Sadasivam and Manickam 1996).

Lipase assay: Lipase activity was detected by the method of Colowick and Kaplan (1955). Emulsion of olive oil and 2% polyvinyl alcohol solution was used as the substrate. The liberated free fatty acids in the enzyme-substrate complex were titrated with 0.02 N NaOH.

RESULTS AND DISCUSSION

Cirrhinus mrigala

The aerobic bacterial population in the gastrointestinal tract of fish mrigal fed different diets on TSA plate are presented in (Table-17). Analyses show that the aerobic bacterial population on TSA plate is maximum in *Cirrhinus mrigala* fed on diet-5 ($1.1x10^6$) followed by fish fed fishmeal based reference diet (0.80×10^6 bacterial cells/g digestive tract) and minimum in *C. mrigala*, fed on raw soybean based diets (0.1×10^6 bacterial cells/g digestive tract). When enumeration of specific enzyme producing bacterial flora was done and it was observed that proteolytic bacterial flora present in all the fish studied and the maximum count was observed in the gut of mrigal fish fed on diet-5^{th} ($5.2x10^5$) followed by fish fed on fishmeal based reference diet ($4.8x10^5$). Densities of amylolytic bacterial flora were observed high in mrigal fed on processed soybean based diets 5^{th} and 6^{th}. The cellulolytic population exhibited maximum densities in mrigal fish fed on processed soybean based diets. Lipolytic bacterial flora was detected in all the fish studied and the maximum population density was however recorded in fish fed on reference diet (1.2×10^3 bacterial cells/g digestive tract).

The intensity of extracellular enzyme production by the bacterial strains isolated from the mrigal fed different diets was assayed qualitatively (Table-18). Maximum protease activity was observed in PSV-1 and PSV-2 strains isolated from mrigal fed on processed soybean based diets (Table-19). Peak specific amylase and cellulase activities were exhibited by bacterial strains PSV-1, PSVI-1, PSVIII-2 and PSV-2 respectively (Table-20&21). Specific lipase activity was found to be maximum in FM-1, a strain isolated from fish fed fishmeal based reference diet (Table-22).

In the present investigation, the presence of a considerable population of bacterial flora has been found in the gastrointestinal tracts of the *Cirrhinus mrigala* and certain strains exhibit proteolytic, amylolytic, cellulolytic and lipolytic activities.

Catfish, *C. batrachus*

The results of microbial flora in the gastrointestinal tract of catfish, *C. batrachus* fed different diets on TSA plate are presented in (Table-11). Results of our experiment indicates that the microbial flora on TSA plate was maximum in *C. batrachus* fed on reference diet ($1.2x10^6$) followed by fish fed on diet-5 (0.90×10^6 bacterial cells/g digestive tract), minimum number of bacterial population was observed in catfish fed on raw soybean based diets- 3&4 (0.2×10^6 bacterial cells/g digestive tract) respectively (Table-12). When enumeration of specific enzyme producing bacterial flora was done and it was observed that proteolytic, amylolytic

and lipolytic bacterial flora present in all the catfish studied and the maximum count was observed in the gut of magur fed on reference diet ($5.1x10^5$, $1.05x10^3$, $4.2x10^3$) followed by fish fed on diet-5 ($4.4x10^5$, $1.02x10^3$, $3.6x10^3$) respectively (Table-12).The cellulolytic population exhibited maximum densities in catfish, magur fed on processed soybean based diets. The intensity of extracellular enzyme production by the bacterial strains isolated from the magur fed different diets was assayed qualitatively (Table-11). Maximum protease and lipase activities were observed in FM-1 and FM-2 strains isolated from magur fed on reference diet (Table-13&16). Peak specific amylase and cellulase activities were exhibited by bacterial strains PSV-1,PSV-2, PSVI-1, PSVIII-2 and PSV-1, PVS-2 respectively (Table-14&15) on processed soybean based diets.

In the present investigation, the presence of a considerable population of bacterial flora has been found in the gastrointestinal tracts of the *C. batrachus* and certain strains exhibit proteolytic, amylolytic, cellulolytic and lipolytic activities.

Proteolytic bacteria were detected in the gut of all the fish examined in our study. However, assay of extracellular protease activity of the bacterial isolates showed highest value in FM-1, a strain isolated from magur fed on reference diet. The occurrence of proteolytic bacteria in the gut of fish seems to support the presence of a diet dependent microbial population in fish.

Proteolytic bacteria were detected in the gut of all the fish examined in our study. However, assay of extracellular protease activity of the bacterial isolates showed highest value in PSV-2, a strain isolated from mrigal fed on diet-5. The occurrence of proteolytic bacteria in the gut of fish seems to support the presence of a diet dependent microbial population in fish. The results of the present study indicate that the microorganisms isolated from the fish digestive tract are capable of producing proteolytic, amylolytic, cellulolytic and lipolytic enzymes. Kawai and Ikeda (1972) and Shcherbina et al. (1976) reported adaptive changes in the activity of proteolytic enzymes in common carp (*Cyprinus carpio*) in relation to the type of diet. Das and Tripathi (1991) observed optimum protease activity between pH 7.6 and 8.4 inboth the fingerling and adult grass carp, and among fingerlings the activity increased proportionately with higher amount of protein in diet up to a limit.

Das and Tripathi (1991) reported high amylase activity in the gastrointestinal tract of grass carp, which appeared to be the result of its omnivorous feeding habit. Sarbahi (1951), Dhage (1968) and Phillips (1969) suggested that amylase activity in the intestine of herbivorous carp is much more intense than in carnivorous fishes, this is in agreement with our study in which we observed very low amylase activity in mrigal fish fed on fishmeal based diet. Amylase is secreted by the entire intestine in the Indian major carps, *Catla catla, Labeo rohita and*

Cirrhinus mrigala, and its activity is high toward the proximal end (Dhage 1968). Though reports on microbial amylase activity in fish gut are scanty, endogenous amylase activity in fish is evident. However, the amylolytic bacteria have been detected in fish guts after24 h of starvation in our study, and it seems that some of the flora forms a persistent population.

Reports on existence of cellulase activity in the digestive system of fish are also scant, and moreover, conflicting, with contradictory results. Microbial intestinal cellulase activity was observed by Das and Tripathi (1991) in grass carp and Saha and Ray (1998) in rohu fingerlings. In the present investigation presence of a considerable population of cellulolytic bacteria and their active role in extracellular cellulase production in the *C. mrigala* fed on processed soybean based diets has been confirmed. Stickney and Shumway (1974) earlier concluded that cellulase activity within fish has resulted from a 'stable' microflora maintained within the digestive tract, irrespective of feeding habit. Our observation is in agreement with this. The results of the present investigation also suggest a possible positive correlation between the food habits and intestinal microbial cellulase activity. Cellulolytic microbial flora was not detected in the gastrointestinal tract of the *C. mrigala* fed on fishmeal based diets showed carnivorous feeding of fish.

All the information available about fish intestinal lipase is about its endogenous source. In the present context, microbial lipolytic activity was studied in the gut of the selected *C. mrigala* fish and maximum lipase activity was shown by a strain named FM-2, isolated from fish fed on fishmeal based diets. Carp normally ingest lipid in their diet and this is known to be a source of energy, essential fatty acids and lipid-soluble vitamins. Al-Hussaini (1949) observed the occurrence of lipase in cyprinids and the activity is more concentrated in the anterior intestine than in the posterior intestine. Lipase activity was concentrated in the anterior one-fifth of the intestine in *Cirrhinus mrigala* and *Labeo rohita* but was found to be totally absent in the entire intestine of *Catla catla* (Dhage 1968).

Table.11 Bacterial strains isolated from fish gut and qualitative extracellular enzyme activity

Fish species	Diets given	Enzyme Activity				
Cat fish		**Bacterial strains**	**Protease**	**Amylase**	**Cellulase**	**Lipase**
	Reference diet	FM1 FM2	+++ +++	++ +	- -	+++ +++
	Diet I	RSI-1 RSI-2	++ ++	+ +	+ +	++ ++
	Diet II	RSII-1 RSII-2	+ +	+ +	+ +	+ +
	Diet III	RSIII-1 RSIII-2	+ +	+ +	+ +	+ +
	Diet IV	RSIV-1 RSIV-2	+ +	+ +	+ +	- -
	Diet V	PSV-1 PSV-2	+++ +++	++ ++	++ +	+++ ++
	Diet VI	PSVI-1 PSVI-2	+++ ++	+ ++	++ ++	+++ ++
	Diet VII	PSVII-1 PSVII-2	+++ +++	++ +	++ ++	++ ++
	Diet VIII	PSVIII-1 PSVIII-2	+++ +++	++ +	++ ++	++ ++

+++, high, ++, moderate; +, low; −, nil.
FM- Fishmeal based, RS-Raw soybean based, PS- Processed soybean based

Table 12. Aerobic bacterial count in fish digestive tract

Fish species	Diets given	Bacterial population per g digestive tract				
Cat fish		**Bacterial count in TSA plate ($\times 10^6$)**	**Proteolytic ($\times 10^5$)**	**Amylolytic ($\times 10^3$)**	**Cellulolytic ($\times 10^3$)**	**Lipolytic ($\times 10^3$)**
	Reference diet	1.2	5.1	1.05	0.02	4.2
	Diet 1	0.4	1.4	0.09	0.10	2.9
	Diet 2	0.3	1.2	0.08	0.08	2.3
	Diet 3	0.2	1.0	0.07	0.08	0.9
	Diet 4	0.2	0.90	0.05	0.09	0.08
	Diet 5	0.9	4.4	1.02	1.09	3.6
	Diet 6	0.4	4.0	0.8	0.9	2.9
	Diet 7	0.3	3.7	0.6	1.01	1.5
	Diet 8	0.3	3.1	0.4	1.03	1.2

Values in same vertical column are not significantly different ($p < 0.05$).

Table 13. Profile of Protease activity in the selected strains in *Cat fish*

Bacterial strains	Protease (U)[a]	Protein (mg/ml)[b]	Specific enzyme activity[c]
FM1	19.9	11.4	1.74
FM2	17.5	10.8	1.62
RSI-1	12.1	11.4	1.06
RSI-2	12.2	10.4	1.17
RSII-1	12.7	12.6	1.01
RSII-2	17.8	16.9	1.05
RSIII-1	18.1	18.3	0.99
RSIII-2	18.8	17.1	1.09
RSIV-1	12.9	14.1	0.91
RSIV-2	12.2	13.5	0.90
PSV-1	18.8	17.9	1.05
PSV-2	15.7	13.7	1.14
PSVI-1	18.9	12.4	1.52
PSVI-2	17.3	11.6	1.49
PSVII-1	15.6	12.2	1.27
PSVII-2	17.9	14.7	1.21
PSVIII-1	16.2	13.0	1.24
PSVIII-2	17.8	14.4	1.23

[a] µg tyrosine liberated per ml of culture filtrate/min
[b] culture filtrate
[c] U/mg protein

Table 14. Profile of Amylase activity in the selected strains in *Cat fish*

Bacterial strains	Amylase (U)[a]	Protein (mg/ml)[b]	Specific enzyme activity[c]
FM1	1.7	5.7	0.30
FM2	0.8	6.2	0.13
RSI-1	1.5	5.9	0.25
RSI-2	1.6	6.1	0.26
RSII-1	1.4	5.9	0.24
RSII-2	1.5	6.2	0.24
RSIII-1	1.2	6.9	0.17
RSIII-2	1.2	6.8	0.18
RSIV-1	0.9	7.1	0.13
RSIV-2	0.7	7.0	0.10
PSV-1	2.8	5.8	0.48
PSV-2	2.1	4.7	0.45
PSVI-1	2.5	5.2	0.48
PSVI-2	1.9	4.3	0.44
PSVII-1	1.8	4.3	0.42
PSVII-2	1.5	3.8	0.39
PSVIII-1	1.9	4.8	0.40
PSVIII-2	2.5	5.2	0.48

[a] µg maltose liberated per ml of culture filtrate/min
[b] culture filtrate
[c] U/mg protein

Table 15. Profile of Cellulase activity in the selected strains in *Cat fish*

Bacterial strains	Cellulase (U)[a]	Protein (mg/ml)[b]	Specific enzyme activity[c]
RSI-1	1.3	5.2	0.25
RSI-2	1.8	6.2	0.29
RSII-1	1.2	5.6	0.21
RSII-2	2.9	8.2	0.35
RSIII-1	2.1	6.9	0.30
RSIII-2	1.9	9.8	0.19
RSIV-1	1.2	6.2	0.19
RSIV-2	1.0	6.5	0.15
PSV-1	3.9	8.2	0.47
PSV-2	5.7	9.2	0.62
PSVI-1	2.3	5.7	0.40
PSVI-2	1.9	8.2	0.23
PSVII-1	2.7	6.8	0.40
PSVII-2	2.8	7.1	0.39
PSVIII-1	1.9	8.8	0.22
PSVIII-2	2.8	9.2	0.34

[a] µg glucose liberated per ml of culture filtrate/min
[b] culture filtrate
[c] U/mg protein

Table 16. Profile of lipase activity in the selected strains in *Cat fish*

Bacterial strains	Lipase (U)[a]	Protein (mg/ml)[b]	Specific enzyme activity[c]
FM1	2.9	2.1	1.38
FM2	4.1	3.2	1.28
RSI-1	1.0	2.2	0.45
RSI-2	1.2	2.6	0.46
RSII-1	1.0	2.3	0.43
RSII-2	0.9	2.7	0.33
RSIII-1	1.1	2.9	0.37
RSIII-2	0.7	3.2	0.21
RSIV-1	0.4	2.6	0.15
RSIV-2	0.5	2.1	0.23
PSV-1	2.6	3.1	0.83
PSV-2	1.8	2.9	0.62
PSVI-1	1.5	3.1	0.48
PSVI-2	2.6	2.8	0.93
PSVII-1	1.7	3.6	0.47
PSVII-2	1.8	2.9	0.62
PSVIII-1	2.5	3.8	0.65
PSVIII-2	1.8	2.9	0.62

[a] micromole fatty acid liberated per min
[b] culture filtrate
[c] U/mg protein

Table 17. Bacterial strains isolated from fish gut and qualitative extracellular enzyme activity

+++, high, ++, moderate; +, low; −, nil.

Fish species	**Diets given**	**Enzyme Activity**				
Cirrhinus mrigala		**Bacterial strains**	**Protease**	**Amylase**	**Cellulase**	**Lipase**
	Reference diet	FM1 FM2	+++ +++	+ +	- -	+++ +++
	Diet I	RSI-1 RSI-2	++ ++	+ +	++ +	++ ++
	Diet II	RSII-1 RSII-2	+ +	+ +	+ +	+ +
	Diet III	RSIII-1 RSIII-2	+ +	+ +	+ +	+ -
	Diet IV	RSIV-1 RSIV-2	+ +	+ +	+ +	- -
	Diet V	PSV-1 PSV-2	+++ +++	++ ++	++ +	+++ ++
	Diet VI	PSVI-1 PSVI-2	+++ ++	+ ++	+++ ++	++ ++
	Diet VII	PSVII-1 PSVII-2	+++ +++	++ +	+++ ++	++ ++

FM- Fishmeal based, RS-Raw soybean based, PS- Processed soybean based

Table 18. Aerobic bacterial count in fish digestive tract

Fish species	**Diets given**	**Bacterial population per g digestive tract**				
Cirrhinus mrigala		**Bacterial count in TSA plate ($\times 10^6$)**	**Proteolytic ($\times 10^5$)**	**Amylolytic ($\times 10^3$)**	**Cellulolytic ($\times 10^3$)**	**Lipolytic ($\times 10^3$)**
	Reference diet	0.8	4.8	0.07	0.01	1.2
	Diet 1	0.3	3.6	0.09	0.10	0.3
	Diet 2	0.3	3.1	0.07	0.08	0.3
	Diet 3	0.2	2.9	0.07	0.08	0.2
	Diet 4	0.1	1.0	0.08	0.09	0.04
	Diet 5	1.1	5.2	0.2	0.3	0.6
	Diet 6	0.4	4.6	0.2	0.2	0.6
	Diet 7	0.4	4.4	0.1	0.3	0.5
	Diet 8	0.4	4.4	0.1	0.3	0.4

Values in same vertical column are not significantly different ($p < 0.05$).

Table 19. Profile of protease activity in the selected strains

Bacterial strains	Protease (U)[a]	Protein (mg/ml)[b]	Specific enzyme activity[c]
FM1	13.2	9.4	1.40
FM2	15.7	10.8	1.45
RSI-1	12.0	10.2	1.18
RSI-2	14.2	16.4	0.87
RSII-1	13.8	11.6	1.19
RSII-2	18.9	15.5	1.22
RSIII-1	17.2	18.9	0.91
RSIII-2	15.7	16.0	0.98
RSIV-1	11.7	13.9	0.84
RSIV-2	10.2	11.4	0.89
PSV-1	21.9	14.8	1.48
PSV-2	23.7	12.7	1.87
PSVI-1	19.3	17.8	1.08
PSVI-2	15.8	11.3	1.40
PSVII-1	14.8	12.5	1.18
PSVII-2	18.9	14.3	1.32
PSVIII-1	19.2	16.8	1.14
PSVIII-2	22.8	15.4	1.48

[a] µg tyrosine liberated per ml of culture filtrate/min
[b] culture filtrate
[c] U/mg protein

Table 20. Profile of Amylase activity in the selected strains

Bacterial strains	Amylase (U)[a]	Protein (mg/ml)[b]	Specific enzyme activity[c]
FM1	0.5	5.7	0.09
FM2	0.8	6.2	0.13
RSI-1	1.5	5.9	0.25
RSI-2	1.6	6.1	0.26
RSII-1	1.4	5.9	0.24
RSII-2	1.5	6.2	0.24
RSIII-1	1.2	6.9	0.17
RSIII-2	1.2	6.8	0.18
RSIV-1	0.9	7.1	0.13
RSIV-2	0.7	7.0	0.10
PSV-1	2.8	5.8	0.48
PSV-2	2.1	4.7	0.45
PSVI-1	2.5	5.2	0.48
PSVI-2	1.9	4.3	0.44
PSVII-1	1.8	4.3	0.42
PSVII-2	1.5	3.8	0.39
PSVIII-1	1.9	4.8	0.40
PSVIII-2	2.5	5.2	0.48

[a] µg maltose liberated per ml of culture filtrate/min
[b] culture filtrate
[c] U/mg protein

Table 21. Profile of cellulase activity in the selected strains

Bacterial strains	Cellulase (U)[a]	Protein (mg/ml)[b]	Specific enzyme activity[c]
RSI-1	1.3	5.2	0.25
RSI-2	1.8	6.2	0.29
RSII-1	1.2	5.6	0.21
RSII-2	2.9	8.2	0.35
RSIII-1	2.1	6.9	0.30
RSIII-2	1.9	9.8	0.19
RSIV-1	1.2	6.2	0.19
RSIV-2	1.0	6.5	0.15
PSV-1	3.9	8.2	0.47
PSV-2	5.7	9.2	0.62
PSVI-1	2.3	5.7	0.40
PSVI-2	1.9	8.2	0.23
PSVII-1	2.7	6.8	0.40
PSVII-2	2.8	7.1	0.39
PSVIII-1	1.9	8.8	0.22
PSVIII-2	2.8	9.2	0.34

[a] µg glucose liberated per ml of culture filtrate/min
[b] culture filtrate
[c] U/mg protein

Table 22. Profile of lipase activity in the selected strains

Bacterial strains	Lipase (U)[a]	Protein (mg/ml)[b]	Specific enzyme activity[c]
FM1	1.8	1.7	1.05
FM2	2.9	3.2	0.91
RSI-1	1.2	2.9	0.41
RSI-2	1.5	3.7	0.41
RSII-1	0.8	3.2	0.25
RSII-2	0.5	3.7	0.13
RSIII-1	0.6	3.9	0.15
PSV-1	1.7	2.1	0.81
PSV-2	1.5	1.9	0.79
PSVI-1	1.2	1.8	0.67
PSVI-2	1.8	2.3	0.78
PSVII-1	0.92	1.3	0.71
PSVII-2	0.81	1.7	0.48
PSVIII-1	0.53	1.3	0.41
PSVIII-2	0.43	2.9	0.15

[a] micromole fatty acid liberated per min
[b] culture filtrate
[c] U/mg protein

CONCLUSIONS

Soybean is found to better protein source which is good candidates to replace the fishmeal meal and enhance growth in different food habits fishes (*Carassius auratus* (gold fish) harbivor, *Cirrihinus mrigala* (mrigal) omnivore and *Clarias batrachus* (Indian magur). Accordingly the enzyme activities were also recorded followed the same pattern as that of growth.

The information generated from the present investigations might contribute towards better feed formulations for fishes at low cost, either by incorporating the appropriate dietary ingredients or by incorporating the enzyme producing bacterial isolates as probiotics.

SUMMARY

In order to study the Effect of replacement of fish meal in commercial aquafeeds by soybean on growth and metabolism of three fish species of different feeding habits, *Carassius auratus* (gold fish) harbivor, *Cirrihinus mrigala* (mrigal) omnivore and *Clarias batrachus* (Indian magur) carnivore were selected Eight diets (1-4 raw soybean based, 5-8 processed soybean based) with 40% protein level were formulated using fish meal, raw and processed soybean as the protein sources. Fish meal based diet was used as the reference/control diet. Growth performance in terms of live weight gain, growth percent gain in body weight and specific growth rate, protein efficiency ratio and nutrient retention increased with each increase in the level of processed full fat soybean in the diets. FCR values were low in fish fed on diets containing processed full fat soybean. Muscle protein was high, while the values of muscle glycogen were low and liver glycogen were high in fish fed on processed full fat soybean containing diets. Specific protease and amylase enzyme activity and viscero-somatic index (VSI) were also significantly ($P<0.05$) enhanced in fish fed on diets containing processed full fat soybean. Body composition of fish was also significantly affected by experimental diets. Accumulation of carcass protein fat and energy were high in fish fed on diets containing processed full fat soybean as the protein source, while no significant ($P<0.05$) variations in ash contents were observed among different treatments, however, carcass o-PO_4 levels were significantly ($P<0.05$) high in fish fed on controlled diet containing fish meal as the major protein source. In general, significantly ($P<0.05$) low values in total ammonia excretion (kg^{-1} BW d^{-1}) and daily amount of reactive phosphate production (kg^{-1} BW d^{-1}) were recorded in fish fed on processed full fat soybean diets, as compared with the fish fed on fish meal and raw soybean containing diets.

SIGNIFICANCE OF WORK

Expanded aquaculture production require more fish feed, which in turn require higher quantities of alternate protein sources to substitute for fish meal. An estimated 1.5 mmt of alternate proteins will be needed just in the next decade to supply global needs. If fish meal supplies decrease, higher amounts will be needed. Soybean is likely to be find more importance to be alternative source of protein in fish diets.

Secondly, as far as cost is concern soybean is found to cheaper as compare to fish meal. Diets based on soybean were given to fishes of different feeding habits. We obtained 50% success to achieve the goal. Two diets out of three were sufficiently increase the weight of fishes hence in this way the cost of third diet can be saved. Further, it will become economically important as not paying so much farmers will get good profit from fish culture.

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