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Effect of experimental diets on the activities of intestinal digestive enzymes of Grass carp, (*Ctenopharyngodon idella*) and Silver carp (*Hypophthylmichthys molitrix*)

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Abstract: A ninety days feeding experiment was directed to compare the effect of Duckweed (*Lemna minor*) and soybean, *Glycine max* (L) meals as a source of protein on the actions of intestinal digestive enzymes of Chinese carps; Grass carp, *Ctenopharyngodon idella* (Valenciennes, 1844) and Silver carp, *Hypophthylmichthys molitrix* (Valenciennes, 1844) fingerlings reared in monoculture and polyculture system. Two 35 % protein experimental diets, F_{SBM} (feed containing 21 % soybean meal) and F_{DW} (feed having 21 % duckweed) as a source of protein were prepared and fed for twelve weeks. At the end of trial, the experimental diets showed a significant effect on the activities of intestinal digestive enzymes. There is significant ($P<0.001$) difference in the intestinal cellulase, protease and amylase enzymes in both Chinese carps. In culture systems, intestinal protease and amylase activities of fingerlings of *C. idella* fed F_{DW} diets were significantly higher as compared to fish fed F_{SBM} diet. In polyculture system, the intestinal amylase activity of *H. molitrix* fingerlings was significantly higher when fish offered F_{DW} diet as compared to F_{SBM} diet. The results of this study indicate the usage of *L. minor* as a protein source in polyculture system enhances the fish digestive capacity than monoculture system.

Keywords: Chinese carps, Digestive enzymes, *Glycine max*, *Lemna minor*

Introduction

Pakistan comes under the list of countries having precious natural resources (Siddiqui & Aslam, 2017) and has acquainted with many interesting alien fish species. e.g. *H. molitrix* and *C. idella* for many objectives like production enhancement, biological control of mosquitoes and harmful weeds and also for sport fishing (Khan *et al*., 2008). Carps are being cultured in many parts of the world including Pakistan (Aslam *et al*., 2016a). Fry stage of Chinese carps; *H. molitrix* and *C. idella* feed on zooplanktons (Santhanam *et al*., 1990). From one of the most intensively cultured fish is *H. molitrix* having much of the Chinese aquaculture production (Tang, 1981; Liang *et al*., 1981). It is introduced in thirty four countries (Li *et al*., 1990). It is having a filter-feeder nature that feeds mostly on phytoplankton (Lazzaro, 1987). *C. idella* is a herbivorous fish in freshwater bodies. It has a very high growth rate and good taste, that's why it is cultured in many countries of the world. It was introduced for the first time in Pakistan in 1994 from China. The objective of its introduction in Pakistan was to enhance fish production and to control aquatic weed in artificial and natural water bodies (Khan *et al*., 2004). Fish has a lot of food prospective and it can provide liberation from malnutrition, especially in the country like Pakistan. Both *C. idella* and *H. molitrix* are Chinese carps. *C. idella* feeds on terrestrial grasses and aquatic weeds with having short intestine. Grass carp fertilizes the pond as fifty percent of its daily basis food consumed (as double of its body weight) is excreted in a semi-digested form, which helps as a significant basis of organic manure. *H. molitrix* is a shallow filter feeder mostly feed on phytoplanktons (< 0.025 mm size). In comparison with grass carp it has an elongated digestive canal hence feed utilization and digestion is comprehensive (Ashraf *et al*., 2011).

C. idella can reach lengths of one meter and can weigh as high as forty five kg. It has a pale-gray, oblong body, rounded belly, large scales and wide head. *C. idella* have pharyngeal teeth which help in eating aquatic vegetation and are used in controlling aquatic weeds. Teeth may have hooks and are long and serrated. As compare to *C. idella*, *H. molitrix* may reach up to 1.9 m in length with more than 35 kg weight. *H. molitrix* can live up to >20 years. It resembles bighead carp except for its ventral keel which ranges forward to the fore part of the breast to

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the gill casings and its silver color. *H. molitrix* has well adapted gill rakers which help in eating plankton (Kraft *et al*., 2006).

There are many alternative sources of protein (Preston, 1998) and the use of protein from plant sources in fish feed is desirable because of their regular availability and low prices (Fagbenro, 2000). Duckweed is the main example of a tropical resource of feed adept of yielding very higher protein than soybean meal (Skillicorn *et al*., 1993).

To study about digestive enzymes is base to understand the digestion mechanism by how the animal adjusts itself to changing with the nutritional surroundings (Sunde *et al*., 2004). The valuation of the digestive enzyme activity in cultured species may be supportive in feed elements assortment (Lan & Pan, 1993). The herbivorous and omnivorous fishes can digest starchy constituents of plants very efficiently than carnivorous fishes. In omnivore and herbivore fishes, carbohydrate activity (alpha-amylase) is higher than in carnivorous fishes (Fernandez *et al*., 2001). Many studies showed the effect of nourishing habits on alpha-amylase activity founded that omnivorous and herbivorous fish had most high activity of amylase as compared to carnivorous fishes (Fernandez *et al*., 2001; Drewe *et al*., 2004; Horn *et al*., 2006). The proteolytic activity is higher in carnivores than herbivorous sheep. In case of fish this can be measured by the protease activity in homogenates of the fish digestive glands but this may not faultlessly reflect the enzymatic action in the digestive zone (Mukhopadhay 1977; Kawai & Ikeda, 1972).

Cellulose is formed by plants and reused by microbes. This is the highly abundant organic material in the whole world. Its arrangement is so much complicated and very difficult to degrade. Animals don't have enzyme cellulase to digest it directly but do so with the help of microbes like fungi and bacteria (Xiao *et al*., 2002). The cellulose is indigestible, consequently has slight importance as nutritional value in formulating fish feedstuffs. Fishes can't produce cellulase and therefore they are unable to utilize cellulose directly (Li *et al*., 2009). The use of cell contents is by the itemization of the algal cell wall by one of the three mechanisms 1) enzymatic digestion 2) acid hydrolysis 3) mechanical trituration (Bitterlich, 1985) but in stomachless fishes as in filter-feeding silver carp, gut fluid pH is usually above 6 and the absence of cellulase in gut juices also shows that it is very hard for them to itemization of cellulose cell wall by the enzymatic digestion (Bitterlich, 1985).

This study was designed to test the hypothesis that, duckweed as a component of a fish feed has a profound effect on intestinal digestive enzymes of Chinese carps in polyculture than monoculture system. Hence, the main objective of this study was to know the intestinal digestive enzyme activities by using duckweed (*Lemna minor*) by replacing soybean meal (*Glycine max*) in fish feed in comparison with polyculture to monoculture system.

Materials and Methods

Collection of Fish Samples

The experiment was directed for a period of 90 days in glass tanks with dimensions of 60×30×30 cm for monoculture and 90×45×45 cm for polyculture. Large and small fingerlings of silver carp and grass carp (body mass 5.54±0.02 and 9.84±0.08 g and length 7.18±0.01, 11.27±0.03 cm respectively) were purch-ased from Attock and Rawal fish Hatchery Islamabad respectively and transported to Fisheries and Aquaculture lab at Department of Animal Sciences, Quaid-i-Azam University, Islamabad in polythene bags filled with pure oxygen. Then fishes were transferred to the glass tanks containing well oxygenated water. The water of the tank was changed daily with dechlorinated water. Dead fishes were removed with the help of hand net quickly to avoid water fouling. Acclimatization was done for about fifteen days before the starting of the trial. During acclimatization period, the temperature was maintained at 26°C, pH ranged from 7.8, DO was 5.0 mg/L and ammonia was < 0.25 ppm. The experiments were conducted in triplicate and fishes were stocked at a stocking density of 2.5 g/L at the ratio of 15:15.

Collection of Duckweed and Soybean

Fresh duckweed was harvested from Lake View Park, Islamabad with the help of hand net and transported in nylon bags to laboratory. The duckweed were washed, dried and then stored in refrigerator (-20°C) in the form of paste and used whenever required for fish feed preparation. Dry soybean was collected from National Agricultural Research Council (NARC), Islamabad.

Feeding

Two different plant based diets containing soybean and duckweed as a major ingredient with a combination of rice polish, sunflower meal, gluten 30%, vitamin premix, Dicalcium phosphate, Carboxy-methyl cellulose, fish meal, canola meal and wheat

bran. The 35% crude protein practical feeds were formulated following Pearson method (Reddy *et al.,* 2002). For the preparation of 35% protein practical diets all dry components were powdered in a dicer and mixed with oil and H_2O to make a paste. The paste was then delivered through a meat grinder and pellets obtained. The pellets were then oven dried out, packed in plastic jars and stored in refrigerator. Feed was given on a daily basis at 4% of body weight. The proximate composition of the duckweed used in feed preparation is given elsewhere (Aslam *et al*., 2016b, 2017).

Digestive Enzymes Analysis

At the end of trial, fish from each aquarium were captured, sedated with MS222 (60 mgL^{-1}) and the blood was withdrawn from the caudal puncture and centrifuged at 10,000 rpm for 15 mins. Fishes were sacrificed on ice box and their intestines were detached and quickly deep frozen in liquid nitrogen. Intestines were homogenized and then centrifuged at 13,000 rpm for 20 mins at 4°C. Supernatant of samples homogenate and serum were stored at −20°C for digestive enzymes analysis by adopting following methods:

Protease Activity Assay

For determination of Protease activity, Cupp-Enyard, (2008) method was used. The reaction solutions were prepared by adding 5 ml of 0.65% Casein solution (6.5 mg ml^{-1} casein in 50 mM potassium phosphate buffer) in tubes and equilibrate them on water bath at 37°C for 5 min. Then enzyme solution was added. After that, 5 ml of TCA reagent (110 mM TCA, prepared by diluting 6.1N stock 1:55 with distilled water) was added to stop the reaction. Then known volume of enzyme solution were added to each tube even in the blank and incubated for 30 min at 37°C. For tyrosine standard solution, 1.1 mM tyrosine standard stock solution was added in the tubes at following volumes in ml: 0.05, 0.10, 0.20, 0.40, 0.50 and so on than distill water was added for final volume of 2 ml. After 30 min of incubation, all samples and blank solution was filtered and final volume of samples used after filtration was 2 ml. Then sodium carbonate (500 Mm) was added to the samples, standards and blank solution, then Folin's Reagent (0.5 Mm) was added for best results and absorbance of the samples were measured by spectrophotometer at 660 nm.

Amylase Activity Assay

For the determination of Amylase concentration, Bernfield (1951) method was adopted. Biefly 0.5 ml of enzyme was added to the tubes and incubated for 3-4 min at 25°C. After that, 1% starch solution (1.0 g soluble starch in 100 ml of 0.02 M sodium phosphate buffer, pH 6.9 with 0.006 M sodium chloride) was added to the tubes and incubated for 3 min. Then 1 ml dinitrosalicylic acid color reagent (1.0 g 3,5-dinitrosalicylic acid in 50 ml of D.W + 30.0 g sodium potassium tartrate tetrahydrate + 20 ml of 2 N NaOH, final volume 100 ml) was added in each test tube and incubated on boiling water bath for 5 min. After that, 10 ml dist. H_2O was added and mixed well. The absorbance of samples was measured by spectrophotometer at 540 nm.

Cellulase Activity Assay

For the determination of Cellulase enzyme activity, the method of Rajoka & Malik (1997) was used. Briefly, 1 ml of enzyme solution was taken into the tubes and added 1 ml of 0.1 M citrate buffer (0.3708 g of citric acid and 2.932 g of sodium phosphate in 100 ml dist. H_2O, pH 5.0) and 1 ml of 1 % carboxymethyl cellulose (1 g carboxymethyl cellulose in 100 ml dist. H_2O). The test tubes were incubated at 50°C for 30 min. After that, 3 ml of DNS reagent (182 g Na K tartrate + 10 g NaOH +, 10 g DNS + 2 g Phenol + 0.5 g Na_2SO_4; all ingredients were dissolved in 600 ml of dist.H_2O and stored in brown colored bottle at 4°C) was added and boiled on water bath for 15 min. Then 1 ml of 40 % Na K tartrate (40 g of Na K tartarate in 100 ml of dist.H_2O) was added. After cooling at room temperature, absorbance was measured against blank solution (1 ml dist.H_2O added instead of Enzyme solution) at 540 nm by spectrophotometer.

Data Analysis

Data obtained from the feeding trail was expressed as mean ± S.E. The results were analyzed using MANOVA followed by Post Hoc Tuckey Test. Values of $P<0.05$ were considered statistically significant. All statistical calculations were performed using SPSS ver. 23.0.

Results and Discussion

There is significant ($P<0.001$) difference in the intestinal cellulase, protease and amylase enzymes in both Chinese carps (Tab. 1 and 2). The cellulase concentration in the large fingerlings (11.75±0.03 μml^{-1}) and small fingerlings of *C. idella* (10.17±0.02 μml^{-1}) fed F_{DW} and reared under monoculture system

Tab. 1: Effect of experimental diets on the activities of intestinal digestive enzymes (µml^{-1}) of large fingerlings of Chinese carps reared in monoculture and polyculture system.

	Monoculture				Polyculture			
	Silver carp		Grass carp		Silver carp		Grass carp	
Enzymes	F_{SBM}	F_{DW}	F_{SBM}	F_{DW}	F_{SBM}	F_{DW}	F_{SBM}	F_{DW}
Cellulase	5.97 (±0.02^{f})	3.15 (±0.03^{h})	9.67 (±0.02^{d})	11.75 (±0.03^{a})	6.58 (±0.04^{e})	5.65 (±0.03^{g})	10.27 (±0.02^{c})	10.47 (±0.04^{b})
Amylase	158.24 (±0.03^{a})	132.77 (±0.04^{b})	78.68 (±0.05^{e})	80.05 (±0.03^{d})	66.78 (±0.03^{f})	106.80 (±0.04^{c})	50.08 (±0.02^{h})	58.47 (±0.04^{g})
Protease	125.77 (±0.03^{d})	123.56 (±0.04^{g})	124.96 (±0.03^{e})	126.55 (±0.03^{c})	124.19 (±0.03^{f})	123.39 (±0.03^{h})	135.75 (±0.04^{b})	136.66 (±0.03^{a})

Data are represented as mean (±SE), (n=21). The comparison made between F_{SBM} vs $F_{DW.}$ (MANOVA followed by Post Hoc Tuckey Test). $F_{SBM,}$ Basal diet having 21% Soybean Meal; F_{DW}, Basal diet contains 21% Duckweed

Tab. 2: Effect of experimental diets on the activities of intestinal digestive enzymes (µml^{-1}) of small fingerlings of Chinese carps reared in monoculture and polyculture system.

	Monoculture				Polyculture			
	Silver carp		Grass carp		Silver carp		Grass carp	
Enzymes	F_{SBM}	F_{DW}	F_{SBM}	F_{DW}	F_{SBM}	F_{DW}	F_{SBM}	F_{DW}
Cellulase	2.85 (±0.03^{g})	2.48 (±0.01^{h})	8.23 (±0.03^{d})	10.17 (±0.02^{a})	6.26 (±0.02^{e})	5.74 (±0.03^{f})	9.49 (±0.04^{c})	9.97 (±0.01^{b})
Amylase	109.38 (±0.04^{a})	74.95 (±0.02^{e})	75.67 (±0.04^{d})	75.93 (±0.03^{c})	28.37 (±0.03^{h})	74.68 (±0.04^{f})	58.53 (±0.03^{g})	80.08 (±0.02^{b})
Protease	135.76 (±0.03^{b})	123.17 (±0.03^{h})	124.59 (±0.03^{f})	125.87 (±0.03^{d})	125.76 (±0.03^{e})	123.65 (±0.04^{g})	134.07 (±0.03^{c})	137.93 (±0.03^{a})

Data are represented as mean (± SE), (n=21). The comparison made between: F_{SBM} vs $F_{DW.}$ (MANOVA followed by Post Hoc Tuckey Test). $F_{SBM,}$ Basal diet having 21% Soybean Meal; F_{DW}, Basal diet contains 21% Duckweed

was significantly (P<0.001) higher as compared to fish fed F_{SBM} diet. The similar higher concentration of cellulase observed in large fingerlings (10.47±0.04 µml$^{-1)}$ and small fingerlings (9.97±0.01 µml^{-1}) of *C. idella* fed F_{DW} diet and reared in polyculture system. Conversely, the large and small *H. molitrix* showed significant (P<0.001) higher concentration of intestinal cellulose fed F_{SBM} 5.97±0.02 µml^{-1} and 2.85±0.03 µml^{-1} respectively in monoculture system and the same thing happened in polyculture system 6.58±0.04 µml^{-1} and 6.26±0.02 µml^{-1} respectively as shown in figures 1 and 2. In culture systems, intestinal protease and amylase activities of large and small *C. idella* fed F_{DW} diets were also significantly higher as compared to fish fed F_{SBM} diet. The intestinal protease and amylase activity in large and small *H. molitrix* reared under monoculture system on F_{SBM} were significantly higher as compared to fish fed F_{DW} diet. In polyculture system, the intestinal amylase activity of large (106.80±0.04 µml^{-1} and small (74.68±0.04 µml^{-1}) *H. molitrix* were significantly higher when fish offered F_{DW} diet as compared to F_{SBM} diet as shown in figures 3 and 4. However, intestinal protease activity of large (124.19±0.03 µml^{-1}) and small (125.76±0.03 µml^{-1}) *H. molitrix* fed F_{SBM} and reared in polyculture system was significantly higher compared to fish fed F_{DW} diet as shown in figures 5 and 6.

Analysis of digestive enzymes revealed that the cellulase, amylase and protease enzymes concentration were significantly higher (P<0.001) in *C. idella* fed with F_{DW} diet in both polyculture and monoculture system as compared to *H. molitrix* that also showed significantly higher (P<0.001) enzymes concentration with F_{SBM} diet but, low level as compared to *C. idella* in both monoculture as well as in polyculture. The intestinal protease and amylase activity in large and small silver carp reared under monoculture system on F_{SBM} were significantly higher as compared to fish fed F_{DW} diet. In polyculture system, the intestinal amylase activity of large and small silver carp were many fold higher when fish were offered F_{DW} diet as compared to F_{SBM} diet. However, in polyculture system, intestinal protease activity of large and small silver carp fed F_{SBM} was significantly higher compared to fish fed F_{DW} diet. Recent studies on digestive appliances have attentive on determining the abilities of organisms to absorb, hydrolyze and assimilate the significant dietary nutrients, these mechanisms can be inspected by determining the action of digestive enzymes (Guzman *et al*., 2005). Digestive processes in fish are not known properly as that of other vertebrates but the digestive enzymes studies showed similar trend with other most vertebrates. Most literature on the digestive, proteolytic and amylase enzymes activity

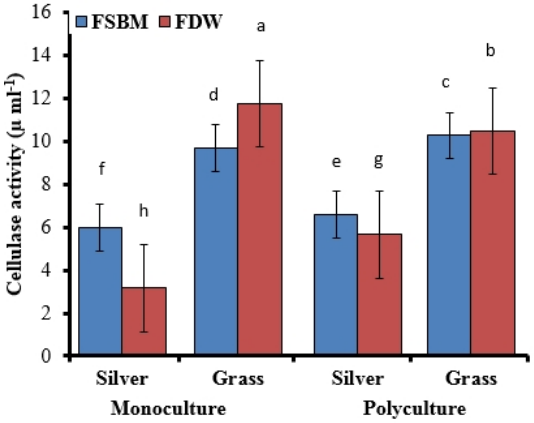

Fig. 1: Cellulase activity in Intestine of large fingerlings of Chinese carps reared in monoculture and polyculture system on duckweed and soybean based diet. Data are represented as mean±SE (n=21). Comparison made: F_{SBM} vs $F_{DW.}$ (MANOVA followed by Post Hoc Tuckey Test). $F_{SBM,}$ Basal diet having 21% Soybean Meal; F_{DW}, Basal diet contains 21% Duckweed.

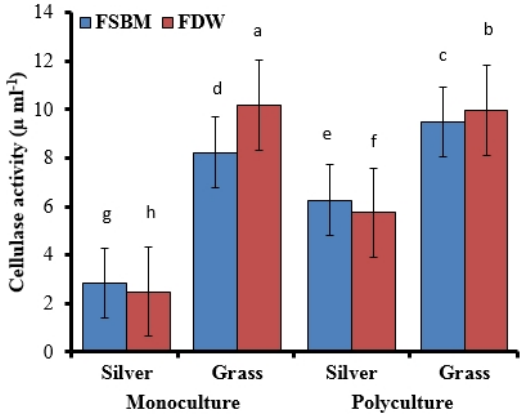

Fig. 2: Cellulase activity in Intestine of small fingerlings of Chinese carps reared in monoculture and polyculture system on duckweed and soybean based diet. Data are represented as mean±SE (n=21). Comparison made: F_{SBM} vs $F_{DW.}$ (MANOVA followed by Post Hoc Tuckey Test). $F_{SBM,}$ Basal diet having 21% Soybean Meal; F_{DW}, Basal diet contains 21% Duckweed.

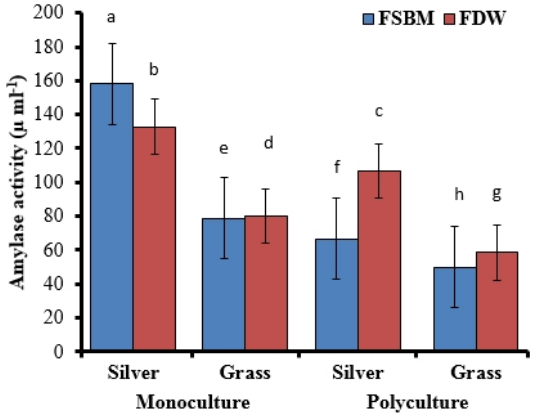

Fig 3. Amylase activity in Intestine of large fingerlings of Chinese carps reared in monoculture and polyculture system on duckweed and soybean based diet. Data are represented as mean±SE (n=21). Comparison made: F_{SBM} vs $F_{DW.}$ (MANOVA followed by Post Hoc Tuckey Test). $F_{SBM,}$ Basal diet having 21% Soybean Meal; F_{DW}, Basal diet contains 21% Duckweed

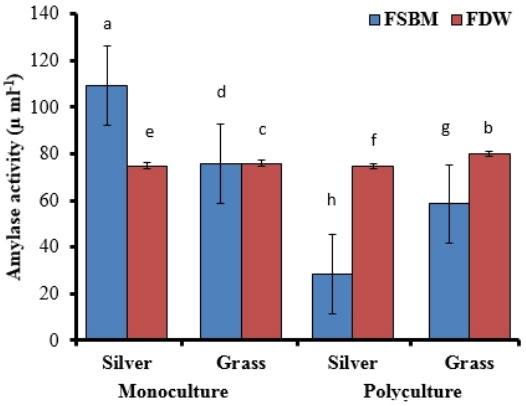

Fig. 4: Amylase activity in Intestine of small fingerlings of Chinese carps reared in monoculture and polyculture system on duckweed and soybean based diet. Data are represented as mean±SE (n=21). Comparison made: F_{SBM} vs $F_{DW.}$ (MANOVA followed by Post Hoc Tuckey Test). $F_{SBM,}$ Basal diet having 21% Soybean Meal; F_{DW}, Basal diet contains 21% Duckweed.

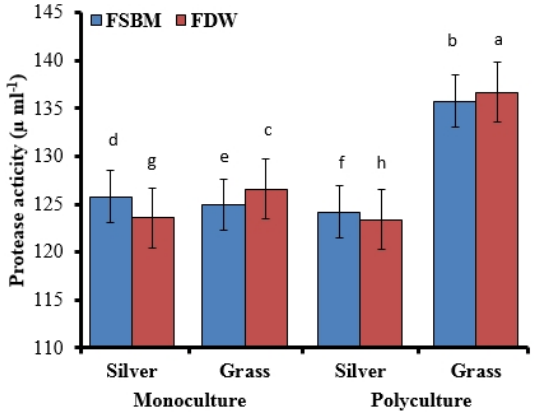

Fig. 5: Protease activity in Intestine of large fingerlings of Chinese carps reared in monoculture and polyculture system on duckweed and soybean based diet. Data are represented as mean±SE (n=21). Comparison made: F_{SBM} vs $F_{DW.}$ (MANOVA followed by Post Hoc Tuckey Test). $F_{SBM,}$ Basal diet having 21% Soybean Meal; F_{DW}, Basal diet contains 21% Duckweed.

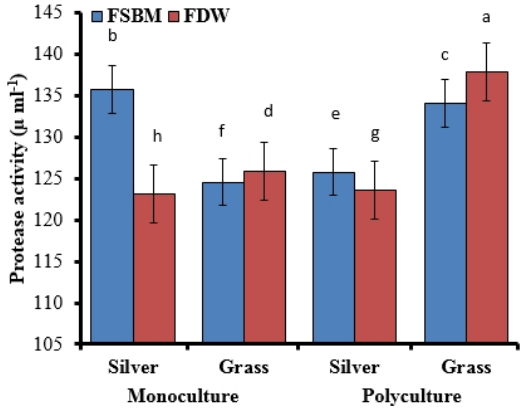

Fig. 6: Protease activity in small fingerlings of juvenile Chinese carps reared in monoculture and polyculture system on duckweed and soybean based diet. Data are represented as mean±SE (n=21). Comparison made: F_{SBM} vs $F_{DW.}$ (MANOVA followed by Post Hoc Tuckey Test). $F_{SBM,}$ Basal diet having 21% Soybean Meal; F_{DW}, Basal diet contains 21% Duckweed.

revealed the capacity of different species to use carbohydrates and proteins (Hidalgo *et al*., 1999) but changes in action of digestive enzymes is pretentious by biochemical arrangement of food and the feeding behavior of fish (Kuzmina, 1996) and studies on herbivorous and omnivorous fishes indicate higher amylase activity as compared with carnivorous fishes (Kuzmina, 1996; Hidalgo *et al*., 1999; Feranadez *et al*., 2001; Drewe *et al*., 2004; Horn *et al*., 2006). Cultured fish feed on the mixture of different ingredients like wheat bran, grains, fishmeal and soybean in small proportion showed that they contain higher levels of carbohydrate as compared to naturally reared carp (Kawai & Ikeda, 1972; Appleford & Anderson, 1996; Keshavanath *et al*., 2002).

Li *et al* (2009) has studied the intestinal action of cellulase in *C. idella* and isolated six different strain of this enzyme, these strains showed different abilities in producing cellulase in the gut of *C. idella* and enabling the fish to digest cellulose present in feed very efficiently.

The high concentration of cellulase, amylase and protease in the intestine of *C. idella* fed *L. minor* in both monoculture and polyculture is due to the fact that it is a herbivore fish and has intestinal microbes that produce cellulase and digest cellulose efficiently as compared to *H. molitrix* that is an omnivore fish and can't produce microbes in its intestine. The concentration of serine protease is higher in fish fed duckweed and suggests that duckweed contains no trypsin inhibitor while soybean meal contains many antinutritional factors (Cromwell, 1999). However, there is no much previous study on effect of duckweed on digestive enzymes on these selected fish species, thus the present study is the first report on this aspect. Further studies are warranted to study the usage of duckweed in fish feedstuff in other important food fishes.

The better and profound positive effect of duckweed as a cheap basis of houseplant origin protein in fish feedstuff by replacing an expensive source of plant protein, soybean meal on digestive enzyme activities are the evident facts which clearly indicate the use of duckweed in fish feed formulation for good fish growth performance in a cost-effective way.

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