International Journal of Food Science and Nutrition
www.foodsciencejournal.com
ISSN: 2455-4898
Received: 25-12-2022, Accepted: 12-01-2023, Published: 28-01-2023
Volume 8, Issue 1, 2023, Page No. 31-37

Effects of graded level replacement of fish meal by protein hydrolysate on growth performance and survival of common carp (*Cyprinus carpio* var. *Communis*) fingerlings

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Abstract
The present study was conducted to evaluate the replacement of fishmeal with protein hydrolysate in the diet of common carp fingerlings. A 60-days experimental trial was conducted to evaluate the effects of graded level replacement of fish meal by protein hydrolysate on growth performance and survival of common carp fingerlings. The protein hydrolysate was prepared by adding enzyme (Papain) to chicken viscera collected locally. Five iso-nitrogenous (CP:35%) and iso-caloric (3900Kcal/kg) diets *viz* Treatment T_0, T_1, T_2, T_3 and T_4 were formulated in which fish meal were replaced with protein hydrolysate at the rate of 0.0%, 10%, 15%, 20% and 25% respectively. An experimental fish (common carp fingerlings) of average weight 4.95 ± 1.05g were selected. Feeding was done @ 5% fish body weight twice daily. The results revealed that the Body Weight Gain, Protein Efficiency Ratio, Specific Growth Rate was higher in the treatment group (T4) as compared to control group. Feed Conversion Ratio was found to be better in the treatment group (T4) as compared to the control group (T0) and against other treatment groups. All the growth parameters showed statistically significant difference ($P< 0.05$) in the treatment group (T4) as against control group (T0). It may be concluded that protein hydrolysate could be added to the diet of common carp fingerlings @25% to replace fishmeal in oreder to bring down the cost of feed without compromising on growth and survival of fish.

Keywords: protein hydrolysate, fishmeal, common carp, low-cost feed, growth

Introduction
Aquaculture has been established as the notable economic source for numerous countries throughout the globe. It helps in raising the living standards by generating employment and extending food supplies. Since 1970, aquaculture has developed at a speed of 7.5 percent each year, exhibiting its significance in worldwide food security (FAO, SOFIA 2020). "Food security" represents the individuals' capacity to acquire protected, adequate, and nutritious food to carry on with a sound and dynamic way of life (WHO, 2017). Perceiving the sector's potential for additional expansion, as well as the magnitude of the environmental difficulties it will encounter as it expands production, necessitates new sustainable aquaculture development plans. Technical advancements in feeds, genetic selection, biosecurity, disease management, and digital innovation are all required to support sustainable aquaculture.

Fish require nutrients for their growth, health, activity, and to stay alive. Nutrition is important in intensive aquaculture, as it is in more traditional types of animal production, because it affects not just production costs but also fish growth, health, and waste output (Gatlin, 2002). Feed expenses account for at least half of total aquaculture production costs, which is dominant because of the monetary cost of protein part in aquafeeds. The most notable repetitive expense in aquaculture is the feed cost and it frequently goes from 60-70% (Singh *et al*., 2006). The essential part of fish feed is protein, both given quality and amount, and hence the costliest feed part. Fish meal is right now utilized as the main protein source in aquafeeds. In many places, the reliance on fish meal for intensive and semi-intensive aquaculture feeds is a significant barrier to the industry's development and intensification. Fish meal supply in the country is variable and the cost is truly expanding. Other than the scarcity of fishmeal, expanding cost of fishmeal has turned into another major issue. Dependence of aquafeeds on fishmeal need to be decreased for sustainable aquaculture (Hardy, 2010).

To support the growth of this activity, Protein hydrolysates that are rich in amino acids can be used as a protein source in the feed. Since amino acid-rich feed consumption aids in the immediate absorption of amino acids into the circulatory system for efficient conversion into body biomass and enhancing fish health, feed supplementation with protein hydrolysates also improves overall performance in fish growth and development.

The use of proteolytic enzymes in the fish diet has ushered in a new era of research. Papain is a cysteine proteinase that breaks down proteins. It's made with papaya leaves, unripe fruit, and papaya latex, a smooth liquid that slimes out green papaya. This enzyme breaks down feed protein particles into amino acids and advances the development of fish by expanding the accessibility of amino acids. Papain is the most potent plant proteolytic enzyme, acting equally well in acid, alkaline, and neutral environments to break down proteins. Other than it, Papain has antiseptic properties and helps in forestalling the unusual multiplication of abnormal microscopic organisms in the digestive system (Ray, 1990). Papain is also involved in the treatment of indigestion as papain transforms protein into peptones, which are more easily absorbed into the bloodstream, thus facilitating protein assimilation and improving indigestion. In addition, papain degrades phytate phosphate and transforms it into a usable form. All plant feed ingredients contain natural phosphorus, which is only 30% available, and 70% as a kind of phytate phosphorus, which is not efficiently utilized by fish (NRC, 1993; Ketola and Harland, 1993). Phosphorus is excreted out in the aquatic environment and then stimulates

the excessive growth of algae and phytoplankton attributing reduced dissolved oxygen and eutrophication (Sugiura *et al*., 1999). In our case, enzyme papain was used for the enzymatic hydrolysis of chicken viscera to convert it into protein hydrolysate.

Materials and methods

Procurement of raw material and preparation of protein hydrolysate: Poultry viscera, which consists of intestine, spleen, gall bladder and connective tissue, were obtained from local market immediately after slaughtering the bird. The tissue was cleaned superficially with tap water and stored in freezer until use. Poultry viscera were homogenized with kitchen blender and mixed with double distilled water in the ratio of 1:2 (PV: DDW) followed with an addition of Papain enzyme to achieve the desired degree of hydrolysis given as under:

Table 1: Hydrolysis conditions for different degrees of hydrolysis.

Enzyme used	Degree of hydrolysis (%)	Enzyme to substrate (E / S) ratio (%)	Time of hydrolysis (min)	Temperature(c)	pH
Papain	15	0.70	60	50	6.5±0.2
Papain	10	0.13	60	50	6.5±0.2
Papain	5	0.02	60	50	6.5±0.2

The protein measurements in the samples were carried out by Kjeldahl method. For all the three degrees of hydrolysis, the incubation time and temperature were same as mentioned in above table 1. The process of hydrolysis was then terminated by heat inactivation of enzyme in a water bath at 90±2ºC for 15 min. Then the mixture was filtrated using Whatman filter paper. Furthermore, the filtrate was then oven dried at 80±2ºC for a period of 48-72 h. The dried filtrate was then stored in air tight conditions for futher use.

Formulation and preparation of experimental diets

The feed formulation was done using Pearson's square method using known values of protein content of ingredients. Feed ingredients were procured from the local market, finely ground into powder using an electric grinder and sieved through standard mesh of 200 mm sieve. The feed was prepared using the basic ingredients which included: rice bran, wheat bran, fish meal, mustard oil cake, vegetable oil, vitamin & mineral mixture. The ingredients were weighed
and mixed in appropriate ratios, as determined by Pearson's square method. Feed was prepared in the form of dry pellets with an optimum protein concentration of 35%. Diet formulation was carried out in wet laboratory (Div. Fish Nutrition & Biochemistry), Faculty of fisheries. The next step in feed preparation involved the thorough mixing of dry ingredients and then water was added to the diet and the ingredients were blended using the kitchen blender to form a paste of the mixture. The contents were mixed uniformly with adequate quantities of water to ensure good consistency of doughs for autoclave. Each diet was pelletized using a hand pelletizer having a mesh size of 1mm through which a dough of mixed ingredients was pushed. The wet pellets were spread over clean papers in open air for drying. The dried pellets were then stored in plastic containers for further use.

Table 2: Diet composition of the experimental diets (g %) fed to *C. carpio* fingerlings during the experimental period

	Ingredients	Control 0%	T10%	T215%	T320%	T425%
1.	Fish meal	25.9g	23.31g	22.02g	20.72g	19.43g
2.	Mustard oil cake	25.0g	25.0g	25.0g	25.0g	25.0g
3.	Rice bran	21.0g	21.0g	21.0g	21.0g	21.0g
4.	Wheat bran	21.0g	21.0g	21.0g	21.0g	21.0g
5.	Vegetable oil	5ml	5ml	5ml	5ml	5ml
6.	Vitamin & Mineralmix	2g	2g	2g	2g	2g
7.	Protein hydrolysate	0g	2.59g	3.88g	5.18g	6.47g
	total	100	100	100	100	100

Procurement of fish

Fingerlings of Common carp, *Cyprinus carpio*, with an average weight of 4.95 ±1.05 g were employed in the experiment. The fish were obtained in June 2021 from the National Fish Seed Farm in Manasbal, district Ganderbal. The fishes were transported to the Faculty of Fisheries, Rangil Campus in transparent polythene bags filled one-third with water and two-third with oxygen. The polythene bags were carried in cardboard boxes to prevent the sunlight exposure. They were carefully placed in fibre glass tanks and left alone for the whole night. The fish were given a modest salt and KMnO4 treatment the next day to ameliorate the handling stress. For a few days, the animal was acclimatised in an aerated environment.

Experimental design

A total number of two-hundred (200) fingerlings were obtained for the present study. They were randomly distributed in five distinct experimental groups, in four replicates, following a completely randomized design. The experiment took place at the wet laboratory of Rangil's Faculty of Fisheries for 60 days. The setup comprised of 20 net-covered plastic circular tubs with a 75-liter capacity. The tubs were rinsed first, then filled with a potassium permanganate solution (4mgL-1) and left overnight. The next day, the tubs were flushed and completely cleansed with clean water. In each of the five experimental groups, 200 fish were dispersed at random. Following a totally randomised design, each group had four replicates. Ten fishes with initial average weight 4.95±1.05g were stocked in each plastic tub with chlorine free tap water. The total volume of the water in each tub was maintained at 75 L throughout the experimental period. Aeration was offered 24 hours a day, seven days a week. Each tub's aeration line was outfitted with an air stone and a plastic regulator to keep the air pressure consistent throughout.

Physico-chemical parameters of water
Water quality parameters *viz*. Temperature, pH, dissolved oxygen, total hardness, ammonia, were recorded during the experimental period as per APHA (2012).

Proximate analysis of diets
Moisture, crude protein, ether extract, and ash were calculated by using automated analyser (Instalab.700).

Growth indices
The fish were sampled at 15-day intervals to determine their body weight. The fish were starved for 24 hours before being weighed. An electronic weighing balance was used to determine the weight. The percentage weight gain, SGR, FCR, PER, and Survival rate were calculated.

Data analysis
The data collected were statistically analysed by using statistical package SPSS version 23 in which data was subjected to one-way ANOVA, and Duncan's multiple range test was used to determine the significant differences between the means, $p<0.05$ was considered as statistically significant. The results were expressed as mean± standard error.

Results
Proximate analysis of Protein hydrolysate
The proximate composition of protein hydrolysate prepared from poultry viscera using papain enzyme is presented in table 3. Moisture, fat, crude protein and ash existed in percentage. All the three degree of hydrolysis (DOH) i.e. 5%, 10%, 15% were used for the preparation of hydrolysate, but only hydrolysate prepared through degree of hydrolysis (15%) were selected to be incorporated in the diets. The hydrolysate prepared through degree of hydrolysis (15%) were selected for diet incorporation due to its reasonable protein content i.e., 22%. The lowest crude protein content (13.1%) was present in hydrolysate prepared using degree of hydrolysis 5%, whereas the crude protein content corresponding to degree of hydrolysis 10% were found to be 16.7%. The highest moisture (13.2%) content was present in hydrolysate corresponding to degree of hydrolysis 5% and lowest moisture content (10.3%) was present in hydrolysate corresponding to degree of hydrolysis 15%. The Fat content were similarly found to be highest (10.8%) and lowest (10.1%) in hydrolysates corresponding to degrees of hydrolysis 5% and 15% respectively. Similarly, ash content was highest (3.2%) and lowest (2.9%) in hydrolysates corresponding to degrees of hydrolysis 5% and 15% respectively. It was also observed during the experiment that greater is the degree of hydrolysis, the finer is the sample texture.
On the basis of proximate composition of three protein hydrolysates, the hydrolysate corresponding to highest degree of hydrolysis (DOH=15%) having higher percentage of crude protein than other two protein hydrolysates (DOH= 5% and 10% respectively) were selected to be used in Common carp feed at four different concentrations (10%, 15%, 20% and 25%) respectively and subsequently fish meal was replaced at same concentration to evaluate effect on growth and survival.

Table 3: Proximate analysis of protein hydrolysate (g/100 g of dry weight) at various

Degree of hydrolysis	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
15%	10.3	22	10.1	2.9
10%	12.5	16.7	10.5	3.1
5%	13.2	13.1	10.8	3.2

Degrees of hydrolysis (DOH)
Physico-Chemical parameters of water: The physico-chemical parameters of water such as temperature (℃), pH, dissolved oxygen (mg L^{-1}), total hardness (mg L^{-1}), total alkalinity (mg L^{-1}) and ammonia (µg L^{-1}) were recorded and average values of all the treatments are presented in Table 4.

Table 4: Physico-chemical parameters of water throughout the experimental period for different experimental groups

Treatments	Temperature (°C)	pH (mg/l)	DO (mg/l)	Total hardness (mg/l)	Total alkalinity(mg/l)	Ammonical nitrogen (µg/l)
	Min – Max (mean)	Min –Max (mean)	Min Max(mean)	Min –Max(mean)	Min –Max(mean)	Min – Max(mean)
Control	18.9-25.5	7.3-8.2	7.5-8.6	154.8-189.9	160.3-180.8	31.3-84.3
Treatment 1	18.9-25.5	7.4-8.2	7.6-8.5	160.3-190.8	159-183.5	31.3-85.3
Treatment 2	18.9-25.5	7.4-8.2	7.6-8.5	159-190.3	161.8-184.8	31.3-85.8
Treatment 3	18.9-25.5	7.3-8.2	7.5-8.4	155.8-191.8	163-186	31.3-83.8
Treatment 4	18.8-25.6	7.3-8.2	7.5-8.6	153.8-189.8	163.8-187.5	31.3-85.3

Table 5: Proximate composition of the diets

chemical composition	control0%	t1 10%	t215%	t320%	t425%
Dry matter (%)	91.86	91.93	92.68	93.09	92.05
Crude protein (%)	35.88	35.85	35.67	35.28	34.39
Lipid (%)	09.34	09.85	09.65	09.33	09.31
Ash (%)	06.01	05.61	05.44	05.12	05.98
Crude fibre (%)	7.05	6.48	6.98	8.08	8.03

Growth performance and body indices
The growth performance and body indices of the experimental groups at the end of the experimental period 60 days of feeding trial are shown in Table 6.

Body weight gain
All of the experimental groups were similar in terms of initial body weight (Table 6). The growth performance of Common carp fingerlings fed diets containing various levels of protein hydrolysate is shown in Table 6 (final body weight, weight gain, specific growth rate, feed conversion ratio, and protein efficiency ratio). The treatment group T4 (12.33±0.08) had the highest body weight gain, while the treatment group T0 (9.69 ± 0.06) had the lowest weight gain, which was significantly different ($p< 0.05$).

Specific growth rate (SGR)
The T4 group's average SGR (2.06±0.01) was significantly higher ($p<0.05$) than the other groups. The control group had the lowest SGR value (1.79±0.0), which was significantly different ($p<0.05$). T1 and T2 had significantly different SGR values than the control group.

Feed conversion ratio (FCR)
Different experimental groups had significantly different FCR (($p<0.05$). The T4 group had the lowest FCR value (2.08±0.01). Control group had the greatest FCR value (2.37±0). T1 and T2 treatment groups are found to be significantly different from control group ($p<0.05$).

Protein efficiency ratio (PER)
The T4 group had the highest PER value (1.37±0.01), which was statistically different ($p<0.05$) from the control group. The control group (1.37±0.14) had the lowest PER value, which was significantly different from the other experimental groups.

Survival
During the 8-week experimental period, no deaths were observed. All of the experimental groups, as well as the control group, had 100% survival rates. The control group and treatment groups were found to be statistically non-significant ($p>0.05$).

Table 6: Growth parameters of different

	Percentage of fish meal replaced by protein hydrolysate				
Parameter	**Control (0%)**	**Treatment 1 (10%)**	**Treatment 2 (15%)**	**Treatment 3 (20%)**	**Treatment 4 (25%)**
IBW(g)	5.05±0.02	5.03±0	5.06±0.01	5.03±0	5.05±0.02
FBW(g)	$14.74±0.07^{a}$	$15±0.07^{b}$	$15.58±0.06^{c}$	$16.38±0.06^{d}$	$17.38±0.08^{e}$
BWG	$9.69±0.06^{a}$	$9.97±0.08^{b}$	$10.52±0.06^{c}$	$11.35±0.05^{d}$	$12.33±0.08^{e}$
SGR	$1.79±0^{a}$	$1.82±0.01^{b}$	$1.88±0.01^{c}$	$1.97±0^{d}$	$2.06±0.01^{e}$
FCR	$2.37±0^{e}$	$2.33±0.01^{d}$	$2.29±0.01^{c}$	$2.19±0^{b}$	$2.08±0.01^{a}$
PER	$1.2±0^{a}$	$1.23±0^{b}$	$1.25±0^{c}$	$1.3±0^{d}$	$1.37±0.01^{e}$
SR	100	100	100	100	100

experimental groups fed different experimental diets at the end of the experiment.

Discussion
The protein hydrolysate was prepared according to the method as described by Elavarasan *et al*., 2013 [11, 12] with modifications. The protein hydrolysate was prepared by the application of papain enzyme which is a proteolytic enzyme. The fish meal was directly replaced by protein hydrolysate to the inclusion levels 10%, 15%, 20%, 25%, and 0% in the control diet. Protein is the main nutrient in fish feed which is costly and to bring down the cost of fish feed, locally available ingredients having reasonable protein content should be incorporated in the diets. The objective of the present study was to utilize animal-based by-products to bring down the cost of production. In order to make nutrients available to the fish, the animal by-products like poultry viscera needs to be hydrolyzed for better digestibility. The enzyme used in the present study was papain which is capable to convert the protein mass into arginine (Gajanan, 2015). Papain is a protein cleaving enzyme and aids in growth and digestion (Ray, 1990). Arginine in its natural form have been found to raise the production of growth hormone (Gajanan, 2015). Raised growth hormone levels as well as high protein(bioactive) content of feed may be the reason for the improvement in the growth parameters in the present study with the inclusion of protein hydrolysates (using papain) in the diets of *Cyprinus Carpio*. Bhaskar *et al*., (2007) [3] reported that visceral waste hydrolysate has a balanced amino acid composition and hence has the potential to be used in the balanced fish diets.
In the present study highest weight gain was recorded in the treatment group T4 (12.33±0.08gm) (Table 6) and the lowest weight gain was observed in the control group T0 (9.69±0.06gm) (Table 6), which was significantly different ($P< 0.05$). The mean of the SGR of the treatment group fed with 25% protein hydrolysate (2.06±0.01 %/day) was significantly higher ($p<0.001$) than the other treatment groups. The lowest SGR value was found in the control group T0 (1.79±0 %/day), which was significantly different ($p<0.001$) than treatment groups T1, T2, T3 and T4. Similar results were also found by Tabinda *et al*., (2013) [37] and they reported that SGR was significantly higher ($p<0.05$) in FM0, FM25 and FM50 as compared to FM75 and FM100 in their study while using chicken intestine at different inclusion levels for fish meal substitution.
The treatment group (T4) had the lowest FCR ((2.08±0.01) in the current study (Table 6). The control group had the greatest FCR (2.37±0) of any group (T0). There is a significant difference ($p<0.05$) in the feed conversion ratio (FCR) of *Cyprinus carpio* fed with protein hydrolysate (25%) as compared to the control group(T0) and against other treatment groups. Similar results were also observed by Tabinda *et al*., (2013) [37], who reported that FCR in fish fed diets FM100 and FM75 was significantly higher ($P<0.05$) and slightly decreased by increasing PBM level in fish diets. Study of Giri *et al*., (2009) in catfish (*Clarias batrachus*) (Linn.), Goda *et al*., (2007) in African catfish (*Clarias gariepinus*) and Shapawi *et al*., (2007) in humpback grouper also supports the present study. They reported relatively low FCR by increasing percentage replacement of FM with chicken intestine. The possible reason for the better FCR in treatment group (T4) could be due to the enhanced digestibility of the protein hydrolysate, the enzymatic hydrolysis releases peptides with better nutritional and functional properties. Egerton *et al*., (2020) [10] found a better FCR (0.88 ± 0.07) for fish groups fed with partly hydrolyzed fish protein hydrolysate (80% plant protein + 5% fishmeal + 10% fish protein hydrolysate) than those of fish groups fed with fish meal (35%) based diet (FCR= 0.92 ± 0.09) and the FCR values for plant-based diet (with 15% fishmeal + 80% plant protein) were found to be (1.04 ± 0.06). The stimulatory effects of FPH (fish protein hydrolysate) on feeding have been found in Atlantic salmon (Refstie *et al*., 2004; Hevroy *et al*., 2005) [34] and Asian seabass (Chotikachinda *et al*., 2013). The present findings are in agreement with those of Rodehutscord and Pfeffer (1995) who found better FCR values by the exogenous application of phytase. Forester *et al*., (1999) found that non-inclusion of phytase doesn't show any improvement in the FCR value.
Protein efficiency ratio (PER) defined as the ratio of weight gain to the protein intake is another parameter analysed in the present study. PER shows how well the protein source in

the diet could provide essential amino acid requirement to the fish. Highest PER value was found in the treatment group T4 (1.37±0.01) (Table 6) which was significantly different ($p<0.001$) from the control group (T0) and other treatment groups. In the present study the PER value was found directly proportional to the levels of protein hydrolysate incorporated in the diets and hence the lowest PER value was found in T0 group (1.2±0.00). Similar results were also found by Tabinda *et al*., (2013) [37] and they reported that PER was significantly higher ($p<0.05$) in FM0, FM25 and FM50 as compared to FM75 and FM100 in their study while using chicken intestine for substituting fish meal. Egerton *et al*., (2020) [10] found a better PER (2.57 ± 0.13) for fish groups fed with partly hydrolyzed fish protein hydrolysate (80% plant protein + 5% fishmeal + 10% fish protein hydrolysate) than those of fish groups fed with fish meal (35%) based diet (PER= 2.48 ± 0.26) and the PER values for plant-based diet (with 15% fishmeal + 80% plant protein) were found to be (2.21 ± 0.13). The possible reason for the better PER in treatment group (T4) may be due to the greater availability of amino acids and shorter chain peptides produced from enzymatic hydrolysis. Dabrowski and Glogoski (1997) reported that addition of proteolytic enzyme exogenously to the fish food show increase in protein content.

There was no mortality in this trial during the course of the 8-week experiment since there was no significant difference between the treatment and control groups (table 6). Water temperature, pH, dissolved oxygen, hardness, and ammonia were all monitored and kept within acceptable limits. The water quality was kept at a comfortable level for the rearing of common carp fingerlings in this study. The physio-chemical parameters were evaluated weekly to ensure that these factors did not impact the rearing of fingerlings, which aided the current investigation in discovering a link between protein hydrolysate replacement percentages and growth indices. All of the physico-chemical characteristics of water, such as temperature, pH, dissolved oxygen, hardness, and ammonia, were found to be within the recommended range for fish (Boyd and Tucker, 1998).

In the present study, it has been shown that chicken viscera protein hydrolysate in general could be incorporated into the diet of *Cyprinus Carpio* upto an inclusion level of 25% for fish meal substitution. Future studies are needed, however, to optimize the level of protein hydrolysate for fish meal replacement in diets of *Cyprinus Carpio* to improve growth performance.

Conclusion

From the present study it may be concluded that chicken viscera protein hydrolysate appears to be a promising and sustainable protein source to replace fishmeal in the feed of *Common carp*. The results demonstrated that chicken viscera protein hydrolysate supplemented diets significantly improved the growth performance of experimental fish *Cyprinus carpio* fingerlings. Supplementation of chicken viscera protein hydrolysate in the feed at 25% (Fish meal direct replacement) inclusion level resulted in better growth performance of the fish in terms of its increased weight gain, low feed conversion ratio, high specific growth rate and protein efficiency ratio. The fish thrives well on feed supplemented by chicken viscera protein hydrolysate with maximum growth and minimum mortality. The final outcome of the study reveals that the fish feed cost can be brought down through the application of cheaper ingredients such as chicken viscera protein hydrolysate which will in turn bring down the cost of production.

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