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In vitro digestibility study of some plant protein sources as aquafeed for carps *Labeo rohita* and *Cyprinus carpio* using pH-Stat method

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Aquaculture, as a promising food industry, is expected to meet the demand for quality food from the increasing human population. As the diet is critical for feeding farm fish, such a faster growth in the industry is destined to create stress in the fishmeal market to supply diets to the tune. In this context, here, we studied the protein content of 20 plant ingredients, including aquatic weeds, cereals, pulses and oil-cakes using micro-Kjeldahl method and evaluated *in vitro* digestibility of these ingredients for rohu *Labeo rohita* and common carp *Cyprinus carpio* using pH-Stat method. The protein contents of water fern, duckweed, almond oil-cake and soybean product were 20.81, 39.75, 47.78 and 57.48%, respectively. Species-specific digestibility was found for the same plant ingredient. The degree of hydrolysis for water fern, duck weed, almond oil-cake and soybean product were 14.17, 4.80, 17.30 and 3.57%, respectively for rohu and 4.58, 6.03, 12.17 and 3.35%, respectively for common carp. This study showed that incorporation of water fern and almond oil-cake in the diet of rohu, and duck weed and almond oil-cake in the diet of common carp are beneficial considering their protein content and digestibility. These are cost-effective, protein-rich feed ingredients for aquafeed.

Keywords: Aquaculture, Aquafeed, *In vitro* digestibility, Plant protein, Rohu

World over, the demand for fish, as an affordable and healthy source of protein, is increasing[1]. Fish consumption by 2030 is estimated at 93.612 million tonnes, with India accounting for 10.74%. Globally, production by aquaculture is 66.6 million tonnes, and Asia accounts for 88.39%, and India 6.3%. By 2030, it is expected to reach 101.2 million tonnes, >60% of fish destined for direct human consumption then. It indicates that aquaculture is the fast growing food-producing sector, and it has potential to meet the increasing demand for quality food. Apparently, such a faster growth would stress the fishmeal market to supply diets to the tune[2]. In India, freshwater aquaculture production contributes about 4.03 million tonnes, 55% of the total fish production, and projected to reach 7.50 million tonnes by 2020[3,4]. Carps account for 85% of freshwater aquaculture production in India[3].

Diets are critical for feeding farmed fish, including breeding. Feed accounts for 40-60% of aquaculture production costs[5]. Protein is the most critical ingredient in fish diets on the basis of cost[6] and growth response[7]. In choosing protein source, fish meal is an option, but expensive and also in high demand. The challenge facing the aquaculture industry is to identify economically viable and environmental friendly alternatives to the fish meal. Thus, the feed industry has given emphasis on the viable utilization of plant sources for formulation of feed. However, plant protein has some limitations due to the presence of antinutritional factors and may require processing to eliminate or reduce such compounds[8]. Moreover, these antinutritional compounds have to be evaluated on the target species to avoid adverse physiological functions in the species, such as inhibiting digestive enzyme activities.

To be a viable alternative feedstuff to fish meal in aquafeed, a candidate ingredient must possess certain characteristics, including wide availability, competitive price, easy handling, shipping, storage and use in feed production. Furthermore, it must possess certain nutritional characters, such as low levels of fiber, starch, especially non-soluble carbohydrates and antinutrients, and have a relatively high protein content, favorable amino acid profile, high nutrient digestibility and reasonable palatability[9].

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A combination of plant-derived feed ingredients will be required to replace fish meal and that supplements such as amino acids, flavourings and possibly exogenous enzymes. The common carp *Cyprinus carpio* and rohu *Labeo rohita* of the order Cypriniformes are intensively used in pond culture. Common carp is an omnivore, bottom feeding species while rohu is herbivore, mid-column feeding species. There is an increasing demand for the cost-effective diets with adequate nutritional value and digestibility for these cultivable species.

In vitro digestibility study is the most useful method to understand the digestibility of feed in fish[10]. *In vitro* protein digestibility techniques provide a fast and cost effective alternative to feeding trails as large numbers of protein sources may be examined rapidly. Those show most promise in terms of digestibility, may be isolated for longer term growth trails[11]. The p*H*-Stat protein digestibility technique has been demonstrated as an accurate method for estimating protein digestibility in salmonids[12], shrimp[13] and marine fish larvae[14]. This assay estimates the proteolytic enzyme hydrolysis of a test protein substrate.

The present investigation aims to evaluate the protein content of easily available plant ingredients and *in vitro* digestibility of these ingredients using two economically important carps, common carp and rohu as test species.

Materials and Methods

Protein source and estimation of protein

Twenty plant protein sources were used for the digestibility study; of these 15 were locally available cereal and pulses, 2 each aquatic weeds and terrestrial plants leaves (Table 1). All cereals and pulses were collected from the local market, Delhi and other plant materials were collected locally. The samples were cleaned properly and dried at 50°C in oven dryer (HiCon Instrument, India) for 30 min. Then ground samples were passed through sieve (500 μ) to obtain fine powder. These samples were stored in air tight containers for further assay.

The protein content of each sample was determined by micro-Kjeldahl method using automated nitrogen estimating system (Pelican Instruments, Chennai, India). The per cent of nitrogen and protein for each sample were analyzed using pre-installed programme (TIAMO 2.1, Metrohm, Switzerland). The calculation was as follows:

$$\text{N}(\%) = \frac{14 \times \text{normality of acid} \times \text{titrant value} \times 100}{\text{Sample weight} \times 1000}$$

Table 1—Degree of hydrolysis (%) of different plant protein source (8 mg mL^{-1}) using crude enzyme extract (0.250 U mg^{-1} protein) of rohu and common carp

Plant source	Protein (%)	Degree of hydrolysis (%)	
		Rohu	Common carp
Coffea arabica (Coffee) pulp	8.66±0.10	3.98±1.75	10.83±1.01*
Trapa natans (Water caltrop)	9.62±0.38	7.37±1.12*	14.06±0.22*
Pennisetum glaucum (Bajra)	10.90±0.39	5.92±1.23*	7.70±1.00*
Oryza sativa (Rice)	13.32±1.64	2.01±0.60	4.68±0.23
Trticum sp. (Bulgur)	16.72±0.27	4.47±0.89	4.02±0.45
Salvinia molesta (Water fern)	20.81±0.10	14.17±0.56*	4.58±1.45
Artocarpus heterophyllus (Jackfruit leave)	21.33±0.13	4.13±0.12	5.13±0.01*
Cajanus cajan (Pigeon pea)	22.37±0.61	2.46±0.40	5.58±0.22*
Cicer arietinum (Chick pea)	24.53±0.01	7.25±0.34*	5.36±0.01*
Cicer arietinum (Bengal gram)	26.70±0.68	3.68±0.11	2.57±0.33
Phaseolus vulgaris (Kidney bean)	28.05±0.09	3.13±0.60	3.46±1.00
Vigna mungo (Black lentil)	28.88±2.98	2.12±0.30	1.34±0.22
Vigna radiate (Green gram)	29.33±0.82	2.90±1.79	3.35±1.34
Lens culinaris (Yellow pigeon pea)	29.61±0.56	4.02±0.01	2.34±0.33
Canavalia ensiformis (Jack bean) leave	30.71±0.90	5.92±1.01*	4.97±0.06
Brassica sp. (Mustard, yellow) oil-cake	33.72±0.36	3.91±0.34	4.91±0.01
Brassica sp. (Mustard, brown) oil-cake	34.16±1.19	1.79±0.01	4.24±0.22
Lemna minor (Duck weed)	39.75±0.47	4.80±1.45	6.03±1.78*
Terminalia catappa (Almond) oil-cake	47.78±0.90	17.30±0.12*	12.17±1.00*
Glycine max (Soybean) product	57.48±0.35	3.57±0.01	3.35±0.01

Crude protein (%) was determined by multiplying nitrogen (%) with 6.25.

Preparation of crude enzyme extract

Labeo rohita, rohu (length: 27.14±1.28 cm, weight: 208.53±29.34 g) and *Cyprinus carpio,* common carp (length: 24.46±1.53 cm, weight: 126.45±23.10 g) were obtained from a local fish market in Delhi. The fish were acclimated separately in grow-out tanks for 15 days. Water temperature and pH ranged from 25-30°C and 7.8-8.5 during this period; dissolved oxygen level was ≥6 mg L^{-1}. Fish were fed with diet containing 40% protein (Table 2) *ad libitum* throughout the study period. Fish were starved for 72 h before sampling for complete evacuation of the digestive tract.

Fish were anesthetized with MS 222 (Sigma, USA), the digestive tract and associated glands were collected from individual fish and cleaned. The digestive tissue of each species was pooled (15 fish each). Pooled sample of both fishes were collected separately. Three replicates (three sets of pooled samples) were used for each species. The tissues were weighed, homogenized in chilled distilled water (1:3 w/v). The homogenate was filtered through cheese cloth pretreated in EDTA (ethylenediaminetetraacetic acid, 0.5%). The filtrate was centrifuged at 10000 x g for 30 min at 4°C and supernatant was collected. This supernatant was called as crude enzyme extract. The crude extract of both fishes were stored separately at –20°C for further use.

Table 2—Ingredients, proximate composition and amino acid profile of diet.

Ingredients (g Kg^{-1})	
Dry fish powder[a]	583.3
Wheat flour[b]	402.7
Cod liver oil[c]	10.0
Vitamin-mineral premix[d]	4.0
Proximate composition (g Kg^{-1})	
Moisture	23.6
Crude protein	438.7
Crude fat	93.2
Total carbohydrate	364.4
Free fatty acid **(**g Kg^{-1} of extracted fat**)**	656.8
Amino acid profile (g Kg^{-1} of protein)	
Histidine	12.0
Isolucine	19.0
Lucine	32.0
Lysine	32.0
Methionine	12.0
Phenylalanine	18.2
Threonine	16.0
Valine	23.0
Arginine	23.0

Source: [a]Local fish market, Delhi, India. [b]Local market, Delhi, India. [c]SAECOD, Cod liver oil (Type B) BP Universal Medicare Pvt. Ltd., Mumbai, India. [d]Supradyn, Bayer Consumer Care AG, Basel, Switzerland

Estimation of protein in enzyme sample

The soluble protein in enzyme extract was estimated using Bradford method[15]. The absorbance was measured at 595 nm using UV-visible Spectrophotometer (Shimadzu, Japan). Protein content was calculated using bovine serum albumin (1 mg L^{-1}) as standard.

Total protease activity

Total protease activity of the crude extract was assayed using azocasein as substrate[16]. The absorbance was measured at 366 nm. Total protease activity was expressed as Activity Units = Abs_{366} min^{-1}mg protein in the reaction $mixture^{-1}$; where, $Absorbance_{366}$ Units = Assay Abs. - Blank Abs.

Trypsin and chymotrypsin activities

Trypsin and chymotrypsin activities were assayed using 0.1 mM BAPNA (benzoyl-arg-*p*-nitroanilide) and SAPNA (succinyl-$(Ala)_2$-Pro-Phe-*p*-nitroanilide) in Tris-HCl (50 mM pH 7.5, 20 mM $CaCl_2$) as substrate[17]. The absorbance was recorded at 410 nm, continuously monitoring the change in absorbance of *p*-nitroaniline release for 3 min at 25°C. Activity units were calculated by the following formula:

$$\text{Activity units} = \frac{\text{Abs 410 nm min - 1} \times 1000 \times \text{mL of reaction mixture}}{\text{8800 X mg protein in reaction mixture}}$$

The activity was expressed in U mg $protein^{-1}$ min^{-1}. The molar extinction coefficient of *p*-nitroaniline is 8800.

***In vitro* digestibility: pH STAT method**

The *in vitro* digestibility assay[18,19] was performed in the p*H* STAT Autotitrator (902 Titrando Metrohm, Switzerland). A 10 g of the substrate suspension (pH 8.0) was taken in a jacketed vessel and 200 µL of crude enzyme extract (0.250 U mg $protein^{-1}$, pH 8.0) was added to start the reaction at 25°C. Sodium hydroxide (0.1N) was used to maintain the pH of the reaction mixture at 8.0. The volume of NaOH consumed to keep the pH constant (8.0) was recorded. The degree of hydrolysis (DH) for the plant ingredient was calculated using the formula:

$$\text{Degree of hydrolysis (\%)} = \frac{B \times N_B \times 1.4 \times S\% \times 10}{8 \times 100}$$

where, B=mL of 0.1 N NaOH consumed to maintain the reaction mixture at pH 8.0, N_B = normality of the titrant, S% = protein content in the reaction mixture expressed as %.

Results

Proximate protein content of plant ingredients

The proximate protein content of 20 different plant ingredients was estimated by micro-Kjeldahl method (Table 1). Varying protein content was observed in different sources. The ingredients were classified into 3 groups according to the protein content: low (<20% protein), medium (20-40%) and high (>40%). Five ingredients *viz*., coffee pulp (*Coffea arabica*), water caltrop (*Trapa natans*), bajra (*Pennisetum glaucum*), rice (*Oryza sativa*) flour and bulgur (*Triticum* sp.) showed <20% protein content. Common cereals and pulses belonged to the medium range. These included pigeon pea (*Cajanus cajan*), chickpea (*Cicer arietinum*), Bengal gram (*Cicer arietinum*), kidney bean (*Phaseolus vulgaris*), black lentil (*Vigna mungo*), green gram (*Vigna radiate*) and yellow pigeon pea (*Lens culinaris*). The protein content of two aquatic weeds duckweed (*Lemna minor*) and water fern *Salvinia molesta* were 39.75±0.47 and 20.81±0.10%, respectively. The protein content of agricultural by-products mustard oil-cake and almond oil-cake ranged from 34-48%. These were obtained as by-products from oil manufacturing industries. These could be a cheap and useful replacement for the costly animal protein source provided their digestibility and palatability are significantly good. The protein content of locally available soybean (*Glycine max*) product was >40%.

Digestive enzyme activities

The digestive enzyme activities of both fishes used for *in vitro* digestibility study were estimated. Total protease activity was adjusted to obtain an activity of 0.25 U mg protein^{-1}. The final total protease activities were 0.26±0.01 U mg protein^{-1} for common carp and 0.28±0.01 U mg protein^{-1} for rohu. Trypsin and chymo-trypsin activities were 0.21±0.01 and 1.36±0.10 U mg protein^{-1}, respectively for common carp and 0.19±0.01 and 1.59±0.01 U mg protein^{-1}, respectively for rohu.

In vitro digestibility: pH STAT method

The study of degree of hydrolysis (DH) using pH-Stat method showed the suitability of various plant proteins sources for the preparation of diets for carps such as rohu and common carp. Species-specific digestibility was found for the same plant ingredient (Table 1). The degree of hydrolysis ranged from 1.79-17.30 and 1.34-14.06% in rohu and common carp, respectively. In rohu, low (1.79±0.01%) and high (17.30±0.12%) DH were observed for mustard (yellow) and almond oil-cakes, respectively. The DH ranged from 2-8% for all other ingredients. In common carp, the DH ranged from 1.34-5.58% for the commonly available cereals. The highest DH (14.06%) was found for water caltrop. The digestibility of almond oil-cake was 12.17% in common carp. The study showed that DH value of soybean product ranged from 3.35-3.57% for both rohu and common carp in spite of the highest protein content of this plant product.

Discussion

Use of plant ingredients as protein source for production of aquafeed is very promising. Hasan and Chakrabarti[20] reported the nutritional status of various aquatic plants for the preparation of fish feed. The crude protein content of aquatic plants depends on the availability of nitrogen in the culture environment. The protein content of duckweed was found 7-45%[21] and for water fern 16.5%[22]. In the present study, 21-40% protein contents of both water fern and duckweed confirmed their nutritional value for fish. The amino acids such as threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine and lysine were found in duck weed[23]. The amount of lysine was 5-fold higher in duck weed compared to the recommended dose (2.12%) for common carp.

Many studies showed the protein contents of various pulses, like pigeon pea[24] 18.8-21%, chickpea[25] 24.8%, Bengal gram[26] 21.9-23.2%, yellow pigeon pea[27] 25.5% and lentils[27] 29.6%. Rice polish is reported to have 12-18% protein, whereas the protein content of mustard oil-cake is 25-38%[3]. The protein content of coffee pulp[28] is 10-12%.

Edible oil cakes have high nutritional value; protein content ranged from 15-50%. Their composition varies depending on their variety, growing condition and extraction methods. Due to their high protein content, they are used as animal

feed, especially for ruminants and fish[29]. The cake of sweet almond remaining after oil extraction contains 39-47% protein and 10-18% oil, and is used in animal feed[30]. The protein content of almond oil-cake was found around 48% in the present study. Almond has threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine and lysine. Hence, almond oil-cake serves as a rich source of amino acids for fish diet. Furthermore, absence of trypsin inhibitory activity as well as hemagglutinating activity in almonds[31] indicates its high digestibility.

There is scarcity of information on the digestibility of feedstuff in freshwater fishes especially, in carps. Alarcon *et al.*[14] observed the *in vitro* digestibility of different types of fish meal, soybean meal, lupin meal and corn glutten meal in juvenile seabream *Sparus aurata*. In tuna *Thunnus thynnus*, mean values of DH obtained with plant proteins did not exceed 5%, whereas those obtained with animal proteins ranged from 6-10.62%[32]. Kumar *et al.*[19] studied the DH of three different protein sources using enzyme extract of catla *Catla catla*, rohu *Labeo rohita* and silver carp *Hypophthalmichthys molitrix*. The rate of hydrolysis was significantly higher in silver carp compared to others. In rohu, the degrees of hydrolysis were 1.7±0.16, 1.1±0.08 and 2.1±0.13% for soybean meal, silver cup and Chilean fish meal, respectively. In the present study, 6-8 plant ingredients (indicated with* in the table) for rohu and common carp showed >5% digestibility. Species-specific high degree of hydrolysis of some ingredients measured may be explained by combination of factors. This includes difference in solubility and/or buffering capacity of proteins, susceptibility of amino acids to cleavage by alkali proteases (trypsin-chymotrypsin), and the susceptibility of peptide bonds to proteases depends on their accessibility and flexibility. The quantitative level of highly susceptible amino acid in a given protein could determine the extent of enzymatic hydrolysis[14]. Amino acids like aspartic acid, cystine, glycine, leucine, methionine, phenylalanine, tryptophan, tyrosine, valine, alanine, isoleucine, arginine, glutamic acid, histidine, lysine, serine, threonine and proline are found in soybean[33,34]. The study showed that DH value of soybean product was low (3-3.57%) compared to other plant ingredients for both rohu and common carp in spite of the highest protein content. This might be due to the presence of antinutritional factor, soybean trypsin inhibitor (SBTI) in the product. Though the protein contents of water caltrop and bajra were considerably low compared to soybean product, the digestibility was higher in both rohu and common carp.

Pulses and lentils are generally used for human consumption and have high market demand. On the other hand, aquatic weeds which otherwise are considered problematic to the environment and also the almond oil cake, the agro-by product, have considerable amount of protein with high digestibility as reported in the present study. Oilseed production in India has been respectably good[35], and it can be used as alternative and effective protein source for the development of carp feed. High protein content and digestibility of duckweed and almond oil-cake made them as suitable plant protein sources for both carps. Water fern might be used as protein supplement for rohu.

Conclusion

The present study determined the protein content and *in vitro* digestibility of some conventional and non-conventional plant protein sources as potential candidate for aquafeed. Among the different ingredients tested, incorporation of water fern and almond oil-cake in the diet of rohu, and duck weed and almond oil-cake in the diet of common carp are beneficial considering their protein content and digestibility. These are cost-effective, protein-rich feed ingredients for aquafeed.

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