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E Vimalraj
Department of Livestock Products Technology (Meat Science), Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

R Ramani
Department of Livestock Products Technology (Meat Science), Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

V Appa Rao
Department of Livestock Products Technology (Meat Science), Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

M Parthiban
Department of Livestock Products Technology (Meat Science), Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

R Narendrababu
Department of Livestock Products Technology (Meat Science), Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

Corresponding Author
E Vimalraj
Department of Livestock Products Technology (Meat Science), Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

Protein hydrolysate extraction from chicken intestine by enzymatic hydrolysis

E Vimalraj, R Ramani, V Appa Rao, M Parthiban and R Narendrababu

Abstract
Protein hydrolysate extracted from chicken intestine is rich in proteins and peptides having a potential for using as a functional ingredient in animal feeds. Protein hydrolysate was found to have better nutritional and functional properties that could be used as feed additives, which are bioactive and may exert beneficial physiological effects. In this study, protein hydrolysate was extracted from chicken intestine by enzymatic hydrolysis using protease P fungal enzyme. Parameters like yield, physical, chemical properties; proximate analysis, degree of hydrolysis and nitrogen recovery were studied in the extracted protein hydrolysate. The yield of protein hydrolysate was 21.37 ±0.33, pH was 6.6 ± 0.02 and instrumental colour values of Lightness (L*) 37± 0.87, Redness (a*) 0.87 ± 0.03, yellowness (b*) 12.22 ± 0.43, Hue 85.41± 0.42 and Chroma value 12.26 ± 0.42. The protein hydrolysate was having proximate composition of 3.4 ± 0.1 per cent of moisture, 63.63 ± 1.09 per cent of protein, 4.38 ± 0.16 per cent of fat, 19.38 ± 0.57 per cent of carbohydrates and 9.2 ± 0.54 per cent of ash. The degree of hydrolysis of protein hydrolysatewas36.08 ± 0.73 per cent respectively. The nitrogen recovery of the extracted protein hydrolysate was 68.39 ± 1.24 per cent. Thus, the waste from poultry industry i.e. chicken intestine can be effectively utilized by transforming in to protein hydrolysate, which in turn finds possible applications in aqua feed, as a protein supplement and as feed additives for weanlings and can also be further used in the extraction of low molecular bioactive peptides.

Keywords: chicken intestine, enzymatic hydrolysis, protease p, protein hydrolysate

Introduction
Poultry offals are by products obtained from poultry slaughter houses or poultry processing plants. These include parts of poultry carcass such as neck, head, feet, blood, undeveloped eggs, gizzard intestine and feathers. Panda *et al.*, (2000) [20] revealed that the processing and utilization of poultry waste in which poultry intestine contributes about 20 -30% of processing waste that can serve as potential source of proteins, lipids, and tissue proteases. As per global agricultural information network, 0.85 million tons (0.5 kg/bird) of poultry offal is produced per annum. Efficient utilization of these wastes will lead to efficient utilization of byproducts and transformation of wastes into useful products of higher value. The animal byproducts are mostly rich in high quality protein than can be hydrolysed by proteases to obtain bioactive peptides with promising therapeutic, functional and/or nutritional applications, mainly reported in dairy hydrolysate (Choi, Sabikhi, Hassan, & Anand, 2012) [10]. The protein hydrolysates derived from animal by-products have potential applications in food technology as flavourings, functional ingredients, and a good source for amino acids (Cho, Baik, Choi, Hahm, & Kim, 2010; Kumar, Nazeer, & Ganesh, 2012) [7, 17].
Techniques such as enzymatic hydrolysis,autolysis and microbial fermentation are used to prepare protein hydrolysate, of which enzymatic hydrolysis is the most common method and is currently used to produce protein hydrolysates via the addition of numerous enzymes such as alcalase, papain, pepsin, trypsin, bromelain, protease N, protease A, and thermolysin (Chalamaiah *et al.*, 2012) [6]. Several works have been carried out to extract protein hydrolysates from marine flesh and marine wastes. But, the studies on the extraction of protein hydrolysates from chicken intestines are scanty. Hence, the study was carried out with the objective of extracting protein hydrolysate from chicken intestine by enzymatic hydrolysis. The study would throw light on the effective ultilization of poultry waste in to a valuable wealth, which in turn would contribute to the complete utilization of poultry waste.

Materials and Methods
Samples of chicken intestine were collected from local retail outlets. The chicken intestines

were collected from retail outlets were ice packed and immediately brought to the Department of Livestock Products Technology (Meat Science), Madras Veterinary College, Chennai of Commercially available food grade fungal enzyme, protease P "Amano"6 having not less than 60,000 u/g proteolytic activity, was procured from M/s. Amano Pharmaceutical Co. Ltd., Japan.

Methods

The protein hydrolysate was prepared according to the method of Bhaskar *et al.,* (2007) [4]. The chicken intestine of about 500 gms for 6 trails was used after proper cleaning of intestine in running tap water to remove the intestinal contents, dipped in boiling water for 5 minutes and then were cut in to small pieces. These small pieces were sterilized at 121 °C under 15 lbs pressure for 15 minutes. The sterilized chicken intestinal pieces were then cooled and minced in a Waring blender for 5 minutes, followed by centrifugation at 10,500 rpm for 30 minutes at 4 °C. After centrifugation, the contents were separated in to three phases in which the top layer contain fat, mostly of middle layer water and protein rich sediment at the bottom. Both the fat and water layers were discarded and only the protein rich sediment was collected and used for further processing.

Enzymatic hydrolysis of the protein rich sediment

The protein rich sediment was mixed with equal quantity of water (w/v), added with 1.0% fungal protease P (Phycomycetes enzyme) used for hydrolysis at 43±1 °C for 90 min in a hot water bath. After the period of time, hydrolysis was stopped by heating the mixture kept at 85 °C for 5 minutes. The hydrolysate was centrifuged at 11,000 rpm for 20 minutes at 15 °C and the supernatant was collected. The collected supernatant containing protein hydrolysate was lyophilized and used for further studies.

Protein hydrolysate from chicken intestine

The protein hydrolysate obtained was used to assess the yield, physical, chemical characteristics, degree of hydrolysis and nitrogen recovery.

Yield of protein hydrolysate

The yield of chicken intestinal hydrolysate were calculated by determining the weight of hydrolysate and freeze-dried product as a percentage of total weight of intestines used (500 gms) per trail.

$$\text{Yield} = \frac{\text{Weight of hydrolysate in dried form (g)}}{\text{Total weight of intestines (g)}} \times 100$$

Physical Analysis

pH

The pH of the protein hydrolysate was measured using a digital pH meter (Cyberscan pH 510, Merck). The pH was recorded by immersing the combination glass electrode of digital pH meter into the hydrolysate. The pH meter was pre-calibrated using standard solution with pH 7.0 as per the user manual instructions prior to measurement.

Instrumental Colour Analysis

Colour of the protein hydrolysate samples were measured using Hunter lab Mini scan XE plus Spectro-colorimeter (Model No. 45/O-L, Reston Virginia, USA) with geometry of diffuse/80 (sphere - 8mm view) and an illuminant of D65/10 deg. They are expressed using the standard Hunter L* a* b* system. L*, a*, b* values (non-dimensional units which refer to the three axes of the system: a lightness axis (white – black, L*); and two axes representing both hue and chroma, one red - green (a*) and other blue – yellow (b*).The instrument was calibrated with black and white tile (L* = 94, a* = 1.10 and b* = 0.6) every time before the colour measurement was taken. The colour was expressed as L (brightness),a*(redness) and b*(yellowness). The hue (relative position of colour between redness and yellowness) and chroma (colour intensity) was calculated as follows.

$\text{Hue} = \tan^{-1}(b^*/a^*)$

$\text{Chroma} = \sqrt{(a^*)2 + (b^*)2}$

Chemical Analysis

Proximate Composition

The proximate composition of the protein hydrolysate samples such as moisture, fat, protein and ash were analysed by following the standard procedure of AOAC (1995) [1]. Fat content was analysed by using SOCS plus (Model SCS 4, Pelican Equipment Pvt. Ltd., Chennai) and protein content was analysed by using KEL plus (Model Classic DX, Pelican Equipment Pvt. Ltd., Chennai) equipments available in the Department of Livestock Products Technology (Meat Science), Madras Veterinary College, Chennai-7.

Determination of carbohydrates (%)

The total carbohydrate content in percentage of the dried hydrolysate powder sample was determined by the method of Bhattacharjee *et al.,* (2013) [5]. This method involved adding the total values of crude protein, ether extract, crude/dietary fibre, moisture and ash constituents of the sample and subtracting it from 100. The value obtained is the percentage carbohydrate constituent of the sample.

% carbohydrate = 100 - (% moisture + %protein + % fat + % ash)

Protein estimation by lowry's method

Protein content in the hydrolysate was determined out according to the method of Lowry *et al.* (1951) [18] as given below:

Reagents

A. Copper sulphate solution (1%w/v) 1.0 g of copper sulphate was dissolved in distilled water and made up the volume to 100ml.
B. Sodium potassium tartrate solution (2% w/v) 2.0 g of sodium potassium tartrate was dissolved in distilled water and made up to volume to 100ml.
C. Sodium hydroxide solution (0.2M) 8.0g of sodium hydroxide pellet was dissolved in distilled water and made up to the volume to one litre.
D. Sodium carbonate solution (4% w/v) 4.0g of sodium carbonate was dissolved in distilled water and the volume was made to 100 ml.
E. Alkaline reagent To 49ml of reagent C 49ml of reagent D was added. Then 1ml of reagent A was added followed by 1 ml of reagent B. This reagent was prepared fresh. F. Folin's reagent(1N) To 5ml of folin and ciocalteau's phenol reagent. 5ml of distilled water was added. This dilution was done immediately before use.

Procedure
To 0.5 ml of suitably diluted hydrolysed sample, 2.5ml of alkaline reagent (E) was added. The content was mixed rapidly and allowed to stand for 10 minutes at room temperature. Therefore 0.25ml of folin reagent (F) was added and mixed immediately and allowed to stand at room temperature for 30 minutes. The blue colour developed was measured by taking absorbance at 660nm on spectrophotometer against blank of 0.5ml distilled water processed under identical conditions of sample. A standard curve of bovine serum albumin (BSA) from the concentration of 50µg to 250µg was prepared and the protein content of the sample was determined.

Measurement of degree of hydrolysis of proteins
The percentage of solubilized protein in 10% trichloroacetic acid (TCA), in relation to the total protein content of the sample, was measured by the method of Hoyle and Merritt (1994) [15], with modifications. Aliquots of 50 ml of the hydrolyzed protein were mixed with 50 ml of 20% TCA solution to obtain the soluble and insoluble fractions in 10% TCA. After 30 minutes of inactivity, the mixture was centrifuged at 10000 rpm and the soluble protein content of the supernatant was determined by the method of Lowry *et al.*, (1951) [18], modified by Hartree (1972) [12], the result was expressed as mg of protein. Bovine serum albumin was used as the standard. The DH was calculated according to the equation and expressed in percentage.

$$DH\% = \frac{\text{Soluble protein content in 10\% TCA (mg)} \times 100}{\text{Total protein content (mg)}}$$

Nitrogen Recovery (NR)
Nitrogen recovery was used as a solubilization index of nitrogen to describe the production of the hydrolysis. After the enzymatic hydrolysis, the soluble fraction was separated from the insoluble fraction by centrifuging at 14,000 rpm for 15 min. The whole nitrogen was determined in two fractions by Kjeldahl method. The nitrogen recovery was calculated according to the formula given by Benkajul and Morrissey (1997) [3]:

$$\text{Nitrogen recovery (\%)} = \frac{\text{Total nitrogen in supernatant (\%)}}{\text{Total nitrogen in hydrolysed fraction}} \times 100$$

Statistical Analysis
The data was subjected to statistical analysis in SPSS (version 2.0) software with mean ± SE.

Results and Discussion
Yield of protein hydrolysate
The mean ± SE values of average yield of protein hydrolysate are presented. Each trail was done with 500g of intestine and yield of hydrolysate in wet basis was 309.71 ± 3.02 and in dry basis was 21.37 ± 0.33. The yield of protein hydrolysate was 4.28 ± 0.06 per cent were presented in table 1.

Physico-Chemical Characteristics
pH
The mean ± SE values of pH of protein hydrolysate was 6.6 ± 0.02 and it ranged from 6.49 to 6.67 were presented in table 2.

Instrumental Colour Analysis
The mean ± S.E values of lightness, yellowness, redness, hue and chroma are given in Table 2. The mean ± SE values of Lightness (L*) of protein hydrolysate from chicken intestine was 37.53 ± 0.87 and ranged from 34.78 to 39.86. The mean ± SE values of Redness (a*) of protein hydrolysate was 0.87 ± 0.03 respectively and ranged from 0.74 to -1.21. The mean ± SE values of Yellowness (b*) of protein hydrolysate from chicken intestine was 12.22 ± 0.43 respectively and ranged from 11.24 to 13.41. The mean ± SE values of Hue of protein hydrolysate from chicken intestine was 85.41 ± 0.42 respectively and ranged from 84.8 to 86.82.
The mean ± SE values of Chroma of protein hydrolysate from chicken intestine was 12.26± 0.42 respectively and ranged from 11.27 to13.46.

Chemical Analysis
Proximate Composition
The mean ± SE values of proximate composition *viz*, moisture, protein, fat and ash of protein hydrolysate were presented in table 3.

Moisture (%)
The mean ± SE values of moisture content of protein hydrolysate from chicken intestine was 3.4 ± 0.1 per cent respectively and ranged from 3.02 to 3.9 per cent.

Protein (%)
The mean ± SE values of protein content of protein hydrolysate was 63.63 ± 1.09 per cent respectively and ranged from 58.33 to 67.9 percent.

Fat (%)
The mean ± SE values of fat content of protein hydrolysate from chicken intestine was 4.38 ± 0.16 per cent respectively and ranged from 3.9 to 5.1 per cent.

Carbohydrate (%)
The mean ± SE values of carbohydrate content of carbohydrates was 19.38 ± 0.57 per cent respectively and ranged from 15.52 to 22.4 percent.

Ash (%)
The mean ± SE values of ash content of protein hydrolysate was 9.20 ± 0.54 per centrespectively and ranged from 7.2 to 11.3 percent.

Degree of hydrolysis
The mean ± SE values of degree of hydrolysis for protein hydrolysate was 36.08 ± 0.73 per cent respectively and ranged from 34.3 to 38.77 per cent were presented in the table 4.

Nitrogen Recovery
The mean ± SE values of nitrogen recovery of protein hydrolysate was 68.39 ± 1.24 per cent respectively and ranged from 62.8 to 72.41 per cent were presented in table 4.
The yield of protein hydrolysate was 4.28 ±0.06 per cent. In contradiction, Bhaskar *et al.* (2007) [4] prepared protein hydrolysate from sheep intestine with a yield of 6% and Noman *et al.*, (2007) obtained a yield of 17% from Chinese sturgeon muscles hydrolysate. Similar results were observed by Hasan *et al.*, (2018) with a yield of 5.6% protein hydrolysate by using papain enzyme hydrolysis and obtained 5.8% yield from protein hydrolysate prepared by using pepsin

enzyme hydrolysis from *Pangasianodon hypophthalmus* fish.
The mean ± SE values of pH of protein hydrolysate was 6.6 ± 0.02 and it ranged from 6.49 to 6.67.
The results were in accordance with Noman *et al.,* (2018) [19] who studied the functional properties of the protein hydrolysate of Chinese sturgeon with a pH of 6. On contrary, protein hydrolysate from sheep intestine had a pH of 7.01 prepared by Bhaskar *et al.,* (2007) [4] and autolytic degradation of chicken intestine by Jamdar *et al.,* (2008) [16] had a pH of 2.8. The mean ± S.E values of lightness, yellowness, redness, hue and chroma are given in Table 2.

Table 1: Mean ± SE of yield of protein hydrolysate from chicken intestine

Parameters	Results
Intestine (g)	500
Hydrolysate (g)	309.71±3.02
Dried powder (g)	21.37±0.33
Yield %	4.28±0.06

Table 2: Mean ± SE of instrumental colour and pH of protein hydrolysate from chicken intestine

Parameters	Results
pH	6.6 ± 0.02
Lightness (L*)	37.53 ± 0.87
Redness (a*)	0.87 ± 0.03
Yellowness (b*)	12.22 ± 0.43
Hue	85.41 ± 0.42
Chroma	12.26 ± 0.42

Table 3: Mean ± SE values of proximate composition of protein hydrolysate from chicken intestine

Proximate composition	Protein hydrolysate
Moisture (%)	3.4 ± 0.1
Crude protein (%)	63.63 ± 1.09
Fat (%)	4.3 ± 0.16
Ash (%)	9.2 ± 0.5
Carbohydrate (%)	19.38 ± 0.57

Table 4: Mean ± SE values of degree of hydrolysis and nitrogen recovery (%)

Parameter	Results
Protein concentration by Lowry's method	314.67 ± 5.6
Soluble protein content (10% TCA)	113.37 ± 1.7
Degree of hydrolysis	36.08 ± 0.73
Nitrogen recovery (%)	68.39 ± 1.24

The mean ± SE values of Lightness (L*) of protein hydrolysate from chicken intestine was 37.53 ± 0.87 and ranged from 34.78 to 39.86.
These observations were not in agreement with lightness value of protein hydrolysate obtained from *Pangasianodon hypophthalmus* fish by Hassan *et al.,* (2018) [14]. He observed a Lightness value of 78.62 by using papain enzyme hydrolysis while Bhaskar *et al.,* (2007) [4] had Lightness value of 82.54 in the hydrolysate of sheep intestine.
The mean ± SE values of Redness (a*) of protein hydrolysate was 0.87 ± 0.03 respectively and ranged from 0.74 to -1.21. These observations were in agreement with lightness value of protein hydrolysate obtained from *Pangasianodon hypophthalmus* fish by Hassan *et al.,* (2018) [14]. He observed a redness value of -1.27 by using papain enzyme hydrolysis while Bhaskar *et al.,* (2007) [4] had dissimilar results with a Lightness value of -1.38 in the hydrolysate of sheep intestine.
The mean ± SE values of Yellowness (b*) of protein hydrolysate from chicken intestine was 12.22 ± 0.43 respectively and ranged from 11.24 to 13.41. The results were not in agreement with yellowness value of protein hydrolysate obtained from *Pangasianodon hypophthalmus* fish by Hassan *et al.,* (2018) [14]. He observed a yellowness value of 23.66 by using papain enzyme hydrolysis while bhaskar *et al.,* (2007) [4] had similar results in yellowness value of 10.30 in the hydrolysate of sheep intestine.
The mean ± SE values of Hue of protein hydrolysate from chicken intestine was 85.41± 0.42 respectively and ranged from 84.8 to 86.82. The results were in agreement with hue value of protein hydrolysate obtained from *Pangasianodon hypophthalmus* fish by Hassan *et al.,* (2018) [14]. He observed a hue value of 86.51 by using papain enzyme hydrolysis.
The mean ± SE values of Chroma of protein hydrolysate from chicken intestine was 12.26 ± 0.42 respectively and ranged from 11.27 to 13.46. These observations were not in agreement with chroma value of protein hydrolysate obtained from fish by Hassan *et al.* (2018) [14]. He observed a value of 23.83 by using pepsin enzyme hydrolysis. The mean ± SE values of moisture content of protein hydrolysate from chicken intestine was 3.4 ± 0.1 per cent respectively and ranged from 3.02 to 3.9 per cent. These observations were in agreement with moisture content of protein hydrolysate obtained from *Pangasianodon hypophthalmus* fish by Hassan *et al.,* (2018) [14]. He observed a moisture (%) of 3.12 by using papain enzyme hydrolysis and moisture content of 3.20% by using pepsin enzyme hydrolysis while Jamdar *et al.,* (2008) [16] had dissimilar results moisture content of 8.8% in the autolysates of chicken intestine. Similar results was observed in protein hydrolysate from sheep intestine having about 3% moisture prepared by Bhaskar *et al.,* (2007) [4].
The mean ± SE values of protein content of protein hydrolysate was 63.63 ± 1.09 per cent respectively and ranged from 58.33 to 67.9 per cent. These observation were not in agreement with content of protein hydrolysate obtained from *Pangasianodon hypophthalmus* fish by Hassan *et al.,* (2018) [14]. He observed a protein content (%) of 78% by using papain enzyme hydrolysis and protein content of 82.55% by using pepsin enzyme hydrolysis while Jamdar *et al.,* (2008) [16] had dissimilar results protein content of 84% in the autolysate of chicken intestine. Similar results were observed in protein hydrolysate from sheep intestine having about 3% moisture prepared by Bhaskar *et al.,* (2007) [4]. Haldar *et al.,* (2018) [13] had dissimilar results of about 42% protein content in the hydrolysate of freshwater mussel and Noman *et al.,* (2018) [19] had protein content of 79.69% in the hydrolysate from chinese sturgeon contrary to our results.
The mean ± SE values of fat content of protein hydrolysate from chicken intestine was 4.38 ± 0.16 per cent respectively and ranged from 3.9 to 5.1 per cent. These observation were not in agreement with content of protein hydrolysate obtained from *Pangasianodon hypophthalmus* fish by Hassan *et al.,* (2018) [14]. He observed a fat content (%) of 7.5% by using papain enzyme hydrolysis and fat content of 5.12% by using pepsin enzyme hydrolysis giving similar resuts while Bhaskar *et al.,* (2007) [4] had disimilar results in protein hydrolysate from sheep intestine having about 1.2% fat. Haldar *et al.,* (2018) [13] had similar results of about 4.1% fat content in the hydrolysate of freshwater mussel.
The mean ± SE values of carbohydrate content of carbohydrates was 19.38 ± 0.57 per cent respectively and ranged from 15.52 to 22.4 per cent. On contrary, Haldar *et al.,*

(2018) [13] had dissimilar result of about 30.05% carbohydrate content in the hydrolysate of freshwater mussel prepared by using alcalase enzyme.

The mean ± SE values of ash content of protein hydrolysate was 9.20 ± 0.54 per cent respectively and ranged from 7.2 to 11.3 per cent. These observation were not in agreement with content of protein hydrolysate obtained from *Pangasianodon hypophthalmus* fish by Hassan *et al.,* (2018) [14]. He observed ash content of 11.37% by using papain enzyme hydrolysis and ash content of 8.86% by using pepsin enzyme hydrolysis having slight similarity while Jamdar *et al.,* (2008) [16] had dissimilar results ash content of 6.5% in the autolysate of chicken intestine. Dissimilar results were observed in protein hydrolysate from sheep intestine having about 0.93% ash prepared by Bhaskar *et al.,* (2007) [4]. Haldar *et al.,* (2018) [13] had dissimilar results of about 4.1% ash content in the hydrolysate of freshwater mussel.

The mean ± SE values of degree of hydrolysis for protein hydrolysate was 36.08 ± 0.73 per cent respectively and ranged from 34.3 to 38.77 per cent. These observations are not in agreement with DH of protein hydrolysate obtained from *Pangasianodon hypophthalmus* fish by Hassan *et al.,* (2018) [14]. He observed DH of 65.16% by using papain enzyme hydrolysis while Similar results was observed in protein hydrolysate from sheep intestine having about 34.9% DH prepared by Bhaskar *et al.,* (2007) [4].

Haldar *et al.,* (2018) [13] had dissimilar results of about 85% DH in the hydrolysate of freshwater mussel by using alcalase and Noman *et al.,* (2018) [19] had DH of 24.8% in the hydrolysate from Chinese sturgeon in contrary to our results. Yu fu *et al.,* (2018) obtained 26.3% DH in umami protein hydrolysate from bovine muscle by using protease enzyme and protein hydrolysate prepared by Sian egerton *et al.,* (2017) was having DH of 41.47% by using flavourzyme. Which is in contrary to our results.

The mean ± SE values of nitrogen recovery of protein hydrolysate was 68.39 ± 1.24 per cent respectively and ranged from 62.8 to 72.41 per cent. The results are in accordance with Bhaskar *et al.,* (2007) [4] having a nitrogen recovery of 64.60% in the protein hydrolysate prepared from sheep intestine. Similar results Diniz *et al.,* (1997) [9] observed a nitrogen recovery of 42.55 to 74.45% in dog fish hydrolysate. Awuor *et al.,* (2017) [2] observed a nitrogen recovery of 71% from dagaa protein hydrolysate by alcalase hydrolysis.

Conclusion

Chicken intestines are rich in proteins. Low value byproducts like intestines can be effectively utilized for the extraction of bioactive peptides. The protein hydrolysate was having 63.63 ± 1.09 per cent of protein and a yield of 4.28 per cent protein hydrolysate can be recovered from the chicken intestine. It can be used as additives in pet food, aqua feed and as a replacement to the fish meal in the diet ration for swine, Incorporation of protein hydrolyaste may enhance growth rate, feed conversion and is a source of aminoacid for weaned animals.

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