Extraction of Protein Isolates From Grouper (Epinephelus Diacanthus) Filleting Waste Using pH Shift Method; Influence of Process Variables on Recoveries

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Abstract

Recovery of proteins from grouper (*Epinephelus diacanthus*) filleting waste was done using isoelectric solubilization method. During the isolation, influence of process variables such as; temperature, ratio of solvent and homogenate, pH, time of centrifugation and speed of centrifugation on protein yields was studied. It was observed that the homogenate to solvent ratio, centrifugation speed protein recoveries. For isolating the proteins from grouper waste using pH shift process the optimum conditions found were; pH-11.0 in alkaline range, pH 3.0 in acidic range, 120 min extraction time, homogenate to solvent ratio of 1:6 and centrifugation speed of 10000 rpm. During the pH shift processing of grouper proteins myoglobin, total pigments and lipid content of proteins were reduced by 90.77, 70.79 and 82.69%, and 93.91, 79.59 and 82.04%, in alkaline and acidic extraction process, respectively. A significant increase in the lightness and whiteness values of the isolates was observed as compared to the raw material. Protein isolates obtained using acidic solubilisation process were found to be whiter than alkali-aided protein isolate.

Highlights

- Proteins were recovered from grouper filleting waste using pH shift method
- Maximum recoveries were noted at pH 3.0 and pH 11.0
- pH shift process caused significant reduction in myoglobin, pigments and lipids
- Significant increase in the L* values and whiteness of the isolates was observed
- pH shift process was found to be promising in recovering proteins from grouper waste

Introduction

Grouper (*Epinephelus diacanthus*) is one of the important marine fishes that are gaining commercial importance across the world. These fish have high value because of their excellent texture and flavour. The export market for grouper is rapidly increasing in the world especially in the countries like Hong Kong, Singapore and Japan. Due to increased demand in the world market for grouper fish, the processing of this particular fish has been increased in the recent years, resulting in the production of enormous processing waste. These fish processing wastes are a very rich source of nutrients, which often discarded without subjecting them to any treatment or recovery process (Surasani *et al.*, 2019).

Due to the health benefits associated with the fish proteins, there has been a growing interest in increasing their use in food systems (Hultin and Kelleher, 1999). Fish proteins are known for its easy digestibility, better functionality and rich nutritional profile, hence finding a better application in various food systems (Kristinsson and Rasco, 2000). However, there are certain limitations for the fish proteins in food applications in comparison with vegetable and other animal proteins due to high susceptibility to microbial spoilage, lipid oxidation and associated loss in its functionality during processing (Mackie, 1994). Processing methods which can make a quality product by using inexpensive raw material would be much desirable in the current scenario not only to reduce the production cost but also to ease the pressure on existing resources.
One such method for protein isolation from fish processing waste is the isoelectric solubilisation ad precipitation or pH-shift process. The process uses extreme acidic or alkaline pH to separate soluble proteins from insoluble content using centrifugation (Surasani et al., 2017). This method is advantageous over any existing processes like surimi processing due to versatility in application for wide range of complex raw material such as whole fish, fish processing waste or by-products (Gehring et al., 2011). Muscle proteins from processing waste of grouper fish can be recuperated using the pH shift processing, which not only helps in contributing to the food security by providing vital nutrients from low cost raw materials, but also addresses the pollution issues associated with the dumping of these enormous processing wastes. With this view, the study was conducted to recuperate proteins from grouper processing waste using pH shift process and the characteristics of the recovered isolates were studied in detail.

**Material And Methods**

**Raw material**

Grouper fish (*Epinephelus diacanthus*) processing waste i.e. fillet frames were procured from M/S Indo fisheries, fish processing plant, Rajiv Gandhi Nagar Harekala, Mangalore Karnataka and was brought to the laboratory located at the College of Fisheries, Mangalore in sealed insulated polystyrene foam boxes under controlled temperature conditions (< 5 °C). Raw material i.e. grouper fillet frames were kept under chilled storage (5° C) until further use. The fillet frames were manually cut into uniform size pieces followed by mincing and crushing using a laboratory mincer. The fine paste obtained from the mincer was used as raw material. All the preparatory as well as processing operations were done under controlled temperature conditions, preferably in a cold room at < 5 °C.

**Solubilization**

For the extraction process, one part of raw material was added with six parts of deionized water and was homogenized for 60 s (2 × 30 s) using a lab scale tissue homogenizer (Ultra-Turrax, T25, Janke & Kunkel GMBH & Co.KG Staufen, Germany). The resultant homogenate was set to a desired pH using 2 M NaOH or HCl with continuous stirring (MAC instruments, India). Fluctuations in the pH during the extraction process were adjusted by the frequent addition of diluted alkali and acid. Homogenate post pH adjustment was subjected to high speed centrifugation (10000xg for 20 min) in a refrigerated centrifuge (Sorvall ST 16R, Thermo Fisher Scientific, Germany). The supernatant obtained every time after the centrifugation was quantified, filtered using a cloth and analysed for its protein content using Bi-uret method (Robinson and Hogden, 1940). The extract variables tested in this study were were: pH (pH 1.0 – pH 13.0), time of extraction (5 min – 120 min), Homogenate to solvent ratio (1:3–1:20) and centrifugation speed (2,000 rpm – 10,000 rpm). All the analyses were performed in triplicate.

**Precipitation**

The supernatants obtained from the first centrifugation step were carefully separated and were subjected to a pH change using 2 M NaOH and 2M HCl to bring back its pH to the isoelectric point (pH 5.5), indicated by the thick white particles liquid. Post-precipitation step was followed by a second centrifugation step (20 min at
10000xg) to separate the precipitated proteins from the liquid. After every centrifugation step, the supernatant was analysed for its protein content using Biuret method (Robinson and Hogden, 1940).

**Recovery yield**

After the optimization of process parameters, proteins were solubilised at pH with maximum protein solubility and recovery i.e. pH 3.0 and 11.0 and were precipitated at pH 5.5. For calculating the recovery yields, the recovery yield was described as; (a) Protein yield during 1st centrifugation (b) Protein yield during 2nd centrifugation (c) Overall process yield. Protein (a) \( \%_{\text{protein}} = \frac{B}{A} \times 100 \)

(b) \( \%_{\text{protein}} = 100 - \left( \frac{C}{B} \times 100 \right) \)

(c) \( \%_{\text{protein}} = \frac{B - C}{A} \times 100 \)

A: Protein content of the homogenate, B: Protein content of the supernatant after 1st centrifugation, C: Protein content of the supernatant after 2nd centrifugation.

**Characterisation of isolates**

**Proximate composition**

The method described in AOAC (2000) was applied for analysing the protein, lipids, moisture and ash content of the raw material as well as isolates. Multiplication factor of 6.25 was used to obtain the total protein content in the samples.

**Lipid content removal**

Reduction in the lipid content of the isolates was calculated using the equation (dry weight basis):

\[
\text{Lipid reduction (\%)} = \left(1 - \frac{\text{Lipid content of isolate}}{\text{Lipid content of homogenate}}\right) \times 100
\]

**Total myoglobin content removal**

The total myoglobin content of the raw material and isolates was determined using the method described by Chaijan *et al.*, (2006). For this samples (2 g) was added with 40 mM, pH 6.8 phosphate buffer (20 mL) and homogenized for 30 min at 9000 rpm. The supernatant after filtration was mixed with sodium dithionite (0.2 mL of 1% solution) followed by measurement of absorbance at 555 nm.

Myoglobin content (%) = \( (A \times 16.111 \times F \times WS \times 7.6) \times 100 \)

Where, A = absorbance, F = dilution factor, WS = Sample weight (g), 7.6 = milli molar extinction coefficient, 16.111 = molecular weight.

\[
\text{Myoglobin reduction (\%)} = \left(1 - \frac{\text{myoglobin content in isolate}}{\text{myoglobin content in homogenate}}\right) \times 100
\]

**Total pigment content removal**
Total pigment content was determined using the method adopted by Rawdkuen et al., (2009), for which sample (1 g) was added with acetone acid (9 mL) and was incubated for 1 h. After the filtration the sample was read for its absorbance at 640 nm.

Total pigment content (mg/100 g) = \( A_{640} \times 680 \)

(680 is the conversion factor to convert the total pigments and the extracted nitric acid pigments into parts per million of haematin)

\[
\text{Pigment removal\%} = \left(1 - \frac{\text{Pigment content of sample}}{\text{Pigment content of homogenate}}\right) \times 100
\]

**Color analysis**

Color characteristics of samples were analyzed in terms of lightness -L*, redness/greenness-a*, and yellowness/blueness -b* using Color Flex EZ Spectrophotometer (Hunter Lab, Virginia, U.S.) (Surasani et al. 2018). These L*, a*, b* values were used to calculate the overall whiteness of the samples;

\[
\text{Whiteness} = 100 - \left(\sqrt{(100 - L)^2 + a^2 + b^2}\right)
\]

**Functionality of protein isolates**

**Gel preparation and analysis**

Fish protein isolate was made into fine paste using mortar and pestle, kept on ice. Moisture content in the paste was adjusted to 80% (W/W) and added with salt (2.5 g/100 g), followed by fine mixing and stuffing into cellulose casings (2.5 cm diameter). Sealed sausages were incubated at 40 °C for 30 min followed by heating at 90 °C for 20 min (Surasani et al., 2018) and Ingadottir, 2006). Resulting protein gel was cooled rapidly in chilled water (4 °C) for 30 min and stored overnight prior to analysis.

**Gel strength**

Gel strength (g. cm) was measured using a TA-XT plus texture analyzer (Stable Micro Systems, Surrey, UK). Uniformly cut gel samples (2.5 cm) were penetrated using a spherical probe (5 mm) to a depth of 11 mm at 1 mm/s test speed. Gel strength values were obtained using TA-XT plus software (Surasani et al., 2020).

**Water holding capacity**

Water holding capacity (WHC) was determined by mixing sample (1 g) with distilled water (10 mL) followed by vortex mixing and centrifugation at 875xg for 25 min (Foh et al., 2011). The difference in the weight was noted and used to calculate WHC as mL of water absorbed per g sample.

**Folding test**

The folding ability of protein gels was evaluated manually by pressing uniform gel pieces (3 mm) between the fingers (Surasani et al., 2020). The folding ability was assessed using a five point system, based on the breaking and cracking pattern of gels during the test. The scale ranged between 1 and 5; 1 – gel with very weak folding resistance and 5- gel with very strong folding resistance.
Foaming properties

For assessing the foaming stability, protein sample (1 g) was added into distilled water (50 mL) and heated at 60 °C to dissolve the proteins. Foam was created using a homogenizer at 10000 rpm for 5 min. Foam as well as solution were gently separated into a measuring cylinder to measure the volume. The volume ratio of foam to the original liquid volume was calculated and expressed as percentage foaming. Its stability was expressed as the ratio of the initial volume of foam to the volume after 30 min.

Emulsification capacity

For calculating emulsification capacity (EC), 1% protein solution was mixed with 5 mL soybean oil and stirred. This was followed by a high speed homogenization (17000xg for 2 min) and a low speed homogenization (1096xg for 15 min). The fractions formed in the tubes during the centrifugation process were separated and measured separately to calculate emulsification capacity.

\[
\text{Emulsification capacity (mL/100 mL)} = \frac{\text{Emulsified oil (mL)}}{\text{FPI}} \times 100
\]

Statistical analysis

Reading were taken in triplicate and results were expressed as mean ± SD. ANOVA (One-way and two-way analysis of variance) were used to analyze the data and to find the significant differences at 95% level of confidence (p ≤ 0.05) with the help of IBM SPSS v. 20.0 software (SPSS, New York).

Results And Discussion

Protein recovery yields

Influence of pH

The solubility pattern of grouper proteins at different pH values is depicted in Fig. 1. The differences in the solubility of proteins at different pHs was found to be significant (p < 0.05), with maximum solubility at pH 3.0 (55.3%) and pH 11.0 (47.7%), whereas the minimum solubility was recorded at pH 5.0. A sudden change in the solubility pattern was observed between pH 3.0–4.0 and pH 8.0–9.0 with iso-electric point between the pH 5.0 and 5.5. This change is attributed to the presence of more ionisable groups with pKa values between these pH values (Undeland et al., 2002). While studying the croaker protein solubility, Kristinsson and Linag, (2006) found maximum solubility at pH 1.5-3.0 and 11.0–12.0, while the minimum solubility of proteins was observed at pH 5.0-5.5. Similar observations were also reported by Ingadottir, (2004). Common carp proteins had a solubility of 76.3% at pH 2.5 and 87.6% at pH 12.5, during the recovery process using pH shift method (Tian et al., 2017). Similarly sardine proteins had a recovery yield of 73.0% and 77.0% during acid and alkali extraction process (Batista et al., 2007). Shabanpour and Etemadian, (2016), reported that the recovery yields of silver carp proteins and common carp proteins during acid and alkali extraction were 75.1% and 80.8%, 70.2% and 64.5%, respectively.

Influence of extraction times
The solubility pattern of grouper proteins at different extraction times is depicted in Table 1. Increase in the extraction time significantly increased the recovery yields (p < 0.05), that was being maximum at 120 min (58.10 ± 0.40%) and minimum at 5 min (40.70 ± 0.23%). Length of extraction time influences the protein solubility significantly (Batista, 1999) and an increase in the extraction time increased the solubility of proteins from monk fish and hake significantly. Similar observations were noted and reported by Kahn et al. (1974) and Montecalvo et al., (1984).

Table 1

| Extraction time (min) | Recovery (%) | Centrifugation speed (rpm) | Recovery (%) | Weight to volume ratio | Recovery (%) | Centrifugation time | Recovery (%) |
|-----------------------|--------------|----------------------------|--------------|------------------------|--------------|---------------------|--------------|
| 5                     | 40.70 ± 0.23a | 2000                      | 38.76 ± 0.030a | 01:20                  | 22.66 ± 0.35a | 5                   | 55.13 ± 0.24a  |
| 30                    | 50.47 ± 0.32b | 4000                      | 42.51 ± 0.020b | 01:15                  | 24.52 ± 0.17b | 10                  | 58.37 ± 0.24b  |
| 60                    | 53.31 ± 0.19c | 6000                      | 56.13 ± 0.33c | 01:10                  | 30.20 ± 0.33c | 15                  | 63.71 ± 0.28c  |
| 90                    | 57.43 ± 0.24d | 8000                      | 62.49 ± 0.17d | 01:06                  | 46.31 ± 0.20d | 20                  | 63.40 ± 0.06c  |
| 120                   | 57.94 ± 0.40e | 10000                     | 69.73 ± 0.13e | 01:03                  | 58.25 ± 0.30e | 25                  | 63.02 ± 0.17c  |

* Values are represented as Mean ± SD. n = 3. Values in different columns with different superscripts are significantly different.

Table 2

|                     | RAFC (%) | RASC (%) | TPR (%) |
|---------------------|----------|----------|---------|
| ALKI                | 96.14 ± 1.02a | 91.20 ± 0.91b | 87.60 ± 0.86b |
| ACI                 | 94.32 ± 0.90a | 86.46 ± 0.74a | 81.52 ± 0.59a  |

* Values are represented as Mean ± SD. n = 3. Values in different columns with different superscripts are significantly different. RAFC = recovery after 1st centrifugation. RASC = recovery after 2nd centrifugation. TPR = Total process recovery.

**Influence of centrifugation speed**

The solubility pattern of grouper proteins at different centrifugation speeds is depicted in Table 1. Increase in the centrifugation speed significantly increased the recovery yields (p < 0.05), that was being maximum at 10000 rpm (69.73 ± 0.13 %), while minimum recovery was at 2000 rpm (38.76 ± 0.03%). Low speed centrifugation do not create enough centrifugal force on the particles to separate solubilized proteins from
insoluble matter resulting in the formation of gel-like sediment that entraps most of the soluble protein, causing low protein yields. With the increasing centrifugation speed and force rate of sedimentation increases thus by efficient separation of lipids, protein and insoluble matter could be achieved. Kain et al. (2009) observed similar pattern during their studies on peanut protein isolates.

**Influence of homogenate to solvent ratio**

The solubility pattern of grouper proteins at different homogenate and solvent ratio is depicted in Table 1. Increase in the homogenate to solvent (w:v) ratio caused significant increase in the recovery yields ($p < 0.05$), that was being maximum at a ratio of 1:3 (58.25%) and minimum at a ratio of 1:20 (22.66%). Increase in solvent ratio 1:15 and 1:20 increased the overall volume, whereas increasing homogenate weight (i.e. 1:3) caused increase in viscosity and gelation, making it difficult to handle. Homogenate and solvent ratio of 1:6 was found optimum in this study, which gave better protein yield as well as handling conditions. Batista (1999) recorded similar observations while recovering proteins from fish waste. It was reported that using low ratios of homogenate and solvent resulted in high volumes of dilute solutions that were difficult to handle, while using high ratios resulted in high viscous solutions, which was also difficult to handle (Montecalvo et al., 1984 and Batista 1999). It was found that a ratio of 1:10 could give good convenience and also good yields, while a ratio of 1:7 was also an optimal ratio (Montecalvo et al., 1984).

**Influence of centrifugation time**

The solubility pattern of grouper proteins at different centrifugation times on recovery of proteins is given in Table 1. Time of centrifugation had a significant effect on protein solubility and yields, increasing the yield to 63.71% with an increase in time from 5 to 15 min. Further increase in centrifugation time did not influence the recoveries significantly. Panpipat and Chaijan (2017) found that the maximum protein recovery was achieved at a centrifugation speed of 8000 rpm for 20 min, which was also observed by Surasani et al., (2017) during their studies on pangas proteins.

**Characterization of protein isolates obtained from grouper processing waste by pH shift process**

**Proximate composition**

The composition of major elements of protein isolates as well as raw material used was depicted in Table 3. Moisture, protein, fat and ash contents of protein isolates were significantly different from the raw material ($p < 0.05$). The moisture and protein content of the isolates were higher than raw material, while the isolates had lower lipid and ash contents than the raw material. The protein content of grouper processing waste 10.7%, which was 19.47% for alkali aided isolate and 17.77% for acid aided isolates. Marmon and Undeland, (2010) and Vareltzis and Undeland, (2012) reported similar results while studying proteins from whole gutted herring and mussel meat. Acid processed isolates were found to have a little more moisture compared to alkali processed isolates and raw materials. The isolates prepared with acid-aided processing had great tendency towards higher moisture content. The reason for this might be high water holding capacity of the isolates obtained thorough acid-aided process, which resulted in a specific unfolding-refolding pattern during solubilisation as well as precipitation (Nolsøe, 2011).
Table 3
Proximate composition of grouper processing waste and protein isolates prepared by using pH shift method

| Sample  | Moisture (%) | Protein (%) | Lipid (%) | Ash (%) |
|---------|--------------|-------------|-----------|---------|
| Raw material | 69.3 ± 0.81 a | 10.7 ± 0.32 a | 7.52 ± 0.53 b | 12.6 ± 0.72 b |
| ALKI    | 74.53 ± 1.02 b | 19.47 ± 0.87 b | 1.69 ± 0.07 a | 5.5 ± 0.20 a |
| ACI     | 76.5 ± 1.05 b | 17.77 ± 0.63 b | 1.67 ± 0.05 a | 4.13 ± 0.16 a |

* Values are represented as Mean ± SD. n = 3. Values in different columns with different superscripts are significantly different. ALKI; Alkali processed isolates, ACI; acid processed isolates

Lipid removal

During the pH shift processing, lipid content of the proteins was reduced from 7.52% in raw material to 1.69% and 1.67% in alkali and acid processed isolate (Table 4). The removal percentage of lipids in alkali processed isolate was 78.13% and 78.39% in acid processed isolate. Batista et al. (2007) found the lipid reductions of 65.3% and 51.0% in alkali and acid processed sardine proteins, respectively. Dewitt et al. (2007) reported that acid-aided solubilisation of catfish proteins caused a reduction of 74% in lipid content. Higher reduction in lipids of channel catfish was observed in pH shift process than the surimi process (Kristinsson et al., 2005). Kristinsson and Liang (2006) observed that the reduction in lipid content of Atlantic croaker by using alkaline-aided process was four times greater than the conventional process. Higher reduction of lipids in pH shift process because of the separation of membrane lipids, storage lipids and phospholipids from the soluble proteins at extreme acidic and alkaline pH, which gets separated due to density and solubility differences during the centrifugation step (Kristinsson et al., 2005). Among the different processes tested, proteins from alkaline-aided processing had lowest lipid content that might be due to the higher emulsification ability of proteins in alkaline conditions (Kristinsson and Hultin, 2003). The first centrifugation causes a fraction of membrane lipids to get settled as a sediment and neutral lipids to get layered on the top (Hultin and Kelleher, 2000).

Table 4
Lipid, Myoglobin and Pigment removal percentage of isolates

| Sample  | Lipid content (%) | Removal % | Myoglobin content (mg/100 g) | Removal % | Pigment content (mg/100 g) | Removal % |
|---------|-------------------|-----------|-----------------------------|-----------|---------------------------|-----------|
| Raw material | 9.73 ± 0.32 b | -         | 221.03 ± 1.41 c | -         | 283.13 ± 2.59 c | -         |
| ALKI    | 1.69 ± 0.12 a    | 82.69 ± 1.51 a | 20.40 ± 1.42 b | 90.77 ± 0.58 a | 80.07 ± 2.74 b | 70.79 ± 1.89 a |
| ACI     | 1.67 ± 0.13 a    | 82.04 ± 1.31 a | 13.49 ± 1.23 a | 93.91 ± 0.52 a | 55.96 ± 2.22 a | 79.59 ± 1.34 b |

* Values are represented as Mean ± SD. n = 3. Values in different columns with different superscripts are significantly different. ALKI; Alkali processed isolates, ACI; acid processed isolates

Myoglobin and pigment content
Total pigments and myoglobin content of raw material as well as isolates is depicted in Table 4. Content of myoglobin in raw material was higher (p < 0.05) than in protein isolates obtained by isoelectric solubilisation. The myoglobin content of the raw material was 221.03 mg /100 g, and in alkali-aided isolate and acid-aided isolate it was 20.40 and 13.49 mg /100 g, respectively. The myoglobin removal percentage was found to be more in acid-aided isolate (93.91%) followed by alkali-aided isolate (90.77%). The increase in the percentage removal of myoglobin content might be due to higher degradation of muscle protein and its easy and efficient removal (Rawdkuen et al., 2009). Higher and effective removal of myoglobin from sardine and mackerel proteins might be due to alkaline solubilization process Chaijan et al. 2006). However, the other factors that influence the myoglobin removal are type of species, muscle, washing process and storage time.

Total pigments in the raw material was 283.13 mg/100 g while in alkaline and acid processed isolate the content was 80.07 and 55.96 mg/100 g, respectively. Total pigment removal percentage between acidic and alkaline isolates was found to be significantly different. The maximum removal (79.59%) was reported in acid processed isolates while in alkaline isolates the removal percentage was 70.79%. Caroteno-proteins and carotenes contain chromo proteins that contribute to the meat colour (Perez-Alvarez & Fernandez-Lopez, 2006). In muscle foods, myosin and haemoglobin are the major pigments that are responsible for red colour. According to Rawdkuen et al., 2009 more pigment content is extracted from the muscle by alkaline extraction process. During alkaline treatment these extracted pigments may get denatured and could not be co-precipitated at pH 5.5.

Colour characteristics.

Colour characteristics of protein isolates are given in Table 5. Alkali aided isolate was found to have maximum L*(56.60) and whiteness values (54.42%) followed by acid aided isolates (53.46, 52.26%) and raw material (45.75, 44.00%). Kristinsson and Demir, (2003) reported higher whiteness values for the isolates obtained from Spanish mackerel by alkaline processing. Kristinsson et al. (2005) reported similar results for channel catfish protein isolate. Higher lightness values were observed for isolates obtained by pH shift processing of croaker meat. The improvement in whiteness of the protein isolates might be due to degradation of myoglobin under alkaline conditions. Panpipat and Chaijan (2017) stated that the whiteness values of isolates were improved with an increase in its lightness values and a simultaneous decrease in its redness (a*) values. Özyurt et al. (2015) observed that protein isolate obtained by alkali-aided process was whiter and lighter than the isolate prepared from acid aided process (whiteness: 59.50%, L*: 66.56 and whiteness: 56.41%, L*: 60.76, respectively).
Functionality

The gel characteristics and functionality of isolates prepared from grouper processing waste are given in Table 6.

Gel strength

The gels prepared using the isolates in this study were not of very good strength. However, the breaking force (254.23 g) was higher in isolates obtained by alkaline extraction than the isolates from acidic extraction (94.80 g). Similar observations were also made by Panpipat and Chaijan, (2017), who stated that the gels from alkaline processed proteins were superior as compared to its acid counterpart (p < 0.05). This differences might be due to severe denaturation of proteins at extreme acidic pH resulting in poor gel strength. Another reason explained for low gel strength of acid processed isolates was the activity of retained cathepsin L enzymes (Choi and Park, 2002). Moreover, protein gel strength is influenced by various factors like fish species, type and nature of raw material, Extraction method and time, etc. While studying the blue whiting proteins, it was observed that the breaking force for acid processed isolates (149.0 g) was less as compared to the breaking force (331.0 g) of alkaline processed isolates (Nolsøe et al., 2011). Similar observations were also made by Chomnawang and Yongsawatdigul (2013) and Kristinsson et al., (2005), who reported that...
denaturation of proteins was less in alkali extraction as compared to the acid process. Extracting/recovering proteins at extreme acidic pH may cause additional proteolysis and also activates some enzymes that affect the gel forming ability of the proteins (Kristinsson and Liang, 2006).

**Folding ability and expressible moisture**

The folding test score for alkali processed isolates was 2 and for acid aided isolates it was 1. Expressible moisture content of protein isolate gel was 33% in alkali processed isolates and 71% in acid processed isolates. Panpipat and Chaijan, (2017) reported that the unfolding of proteins induced by the acid process was of greater extent than alkaline process, which caused poor networking of the protein gel and low gel strength. Therefore, high water release is shown by the acid processed isolate gel which could not hold water. In general, the increased breaking force was coincidental with low expressible moisture (Chaijan et al., 2006). Further change in the water-binding ability of protein gel may occur during the protein precipitation process, where protein can ameliorate the aggregation.

**Gel color**

Differences in the color characteristics of protein isolates and their gels were insignificant (p > 0.05) in terms of its whiteness values. The whiteness value of gel from alkali and acid processed isolate was 55.01 and 52.93, respectively. Nolsøe et al. (2011) made similar observations, who recorded that the alkaline and acid processed blue whiting isolate gels had a whiteness value of 69.2 and 75.4 respectively. Similar findings were reported for the proteins extracted using pH shift method from silver carp by Paker et al. (2015). Significantly higher whiteness values were observed for isolate gels obtained by pH shift processing of croaker meat (Kristinsson and Liang, 2006), which was reverse in case of isolate gels obtained from from tilapia frame by-products using pH shift method (Chomnawang and Yongsawatdigul, 2013).

**Foaming and emulsification capacity**

The functional properties i.e. foaming capacity, foaming stability and emulsifying capacity were evaluated for the isolates prepared by pH shift method. A significant difference (p < 0.05) was found in the foaming capacity of alkali and acid aided isolates (82.84% and 71.62%, respectively). Foaming capacities of acid and alkaline processed isolates had significant differences (82.84 and 71.62%), while no significant difference was found in its foaming stability values (79.33% and 78.91%). Flexible protein molecules can give good foamability by decreasing the surface tension, whereas ordered globular proteins are difficult to be natured hence causing low formability. During pH shift processing change in pH causes the conformational changes in myofibrillar proteins thus by exposing the hydrophobic residues which get adsorbed at air-water interface (Grahams and Philips, 1976). According to Panpipat and Chaijan, (2017) the protein isolates prepared by isoelectric solubilisation precipitation or pH-shift method from big eye snapper head had excellent foaming properties that can be used in the food industry.

The emulsifying capacity was higher in acid processed isolates (60.82%) as compared to alkali processed isolates (52.5%). Surface hydrophobicity influences the emulsifying properties of salt soluble proteins. Increase in hydrophobicity of cod myosin and associated increase in its emulsification activity during pH shift process indicates that surface hydrophobicity is a measure of emulsifying ability (Kristinsson and Hultin, 2003). In acid aided processing, protein misfolding might have increased the exposure of hydrophobic
residues thus by increasing the surface hydrophobicity and associated increase in its emulsification capacity (Kristinsson and Hultin, 2003). Another factor that contributed would be the dissociation of myosin light chains, leaving more hydrophobic patches exposed, aiding in emulsification (Kristinsson and Hultin, 2003). It was also assumed that increased reactivity of thiol groups would have formed a stable protein network at the interface, resulting in the stabilization of the emulsion.

**Conclusions**

During the recovery of grouper proteins from its filleting waste using pH shift processing, it was observed that protein recovery yields are significantly influenced by the process variables such as homogenate to solvent ratio, centrifugation speed, time of centrifugation and extraction time. Optimum recoveries were observed using pH-3.0 and 11.0, 120 min extraction time, homogenate to solvent ratio of 1:6 and centrifugation speed of 10000 rpm. pH shift process caused significant reduction in the lipid as well as pigment contents of isolates, and also produced the isolates with improved whiteness. pH shift process was found to be promising for processing grouper fish proteins to produce functional and edible proteins from the processing waste. This would not only help in reducing the pollution and operational costs but also contributes to the food security by converting inedible waste into edible proteins. Further in depth research is needed in this area to assess the influence of process on digestibility of recovered proteins.

**Declarations**

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**Contributions**
Conceptualization and methodology: Raju Chikoppa Varadaraju and Vijay Kumar Reddy Surasani; Investigation and data collection were performed by Uzair Shafiqar and Kirankumar Gopalbai Baraiya; Analysis and visualization were performed by Sagar Joshi. The first draft of the manuscript was written by Uzair Shafiq and Vijay Kumar Reddy Surasani. All the authors commented and contributed to the writing and editing of the manuscript. All the authors read and approved the final manuscript.

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**Ethical Approval**

Authors approve that they stick to all the ethical standards and practices related to this work

**Consent to Participate**

Authors give consent related to this work

**Competing interests**

None

**Consent for publication**

All the authors gave consent for publication of this paper in Environmental Science and Pollution Research.

**Data availability**

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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