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Utilisation of isoelectric precipitation to recover fish protein isolate from seafood processing waste

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ABSTRACT

Threadfin bream (*Nemipterus japonicus*) is an important marine fish species used in the preparation of surimi. The waste generated from surimi production contains large quantity of recoverable proteins, which could be utilised for byproduct development. In this study, Isoelectric precipitation (IEP) technique as a method to recover proteins from fish processing waste was attempted. The alkali aided IEP process extracted the fish protein isolate (FPI) efficiently with a yield of 84.13±0.11%. The highest protein yield was recorded at a homogenised sample to water ratio of 1:9. Maximum protein content was found at pH 13 with 19.63±0.52% yield. FPI extracted had higher protein content, less ash and low lipid contents compared to the fish processing waste. The mean yield and protein composition of FPI extracted at different treatments of pH and sample to water ratio were found to be significantly different ($p<0.05$) . Sample to water ratio of 1:9 and pH 13 was found to be the best combination among the different treatments attempted for FPI extraction from threadfin bream processing waste.

Keywords**:** Fish protein isolate, Isoelectric precipitation, Seafood processing waste

Introduction

Pollution from seafood waste disposal is a major issue, creating ecological imbalance and habitat destruction. During the processing of fish, 30-70% of the waste in the form of head, viscera, skin and frames is generated (FAO, 2016). More than 2 million t of waste is generated in India alone from fish processing activities (Geethanjali, 2013). Seafood processing generates both liquid effluents and solid wastes, containing organic and inorganic material. The type and heterogeneity of seafood waste generated is mainly determined by the variety and extend of processing involved. Before taking a decision on mode of waste disposal, the type and variety of the waste needs to be considered as it is expected to affect the efficiency of the disposal process (Mekpiroon *et al.,* 2016). According to Mishra *et al*. (2015) waste generated during the processing of fish can act as a source of energy, livestock feed, fertilisers, liming components for soil, collagen and gelatine. Wastewater obtained from the first washing/rinsing in surimi production contains high concentrations of proteins, non-protein nitrogen, fat and ash. After processing, the waste obtained can be also used in the production of different value added products. Under-utilisation of these byproducts leads to loss of potential revenue as well as increase in the cost of disposal.

Isoelectric precipitation of protein is based on the differences in solubility of muscle proteins in water at different pH values (Marmon, 2012). The isoelectric point (pI) of a protein is the pH at which the net charge on the protein is zero. At the isoelectric point, the proteins tend to aggregate and precipitate because there is no electrostatic repulsion to keep them apart. Each protein has its own isoelectric point because of the different amino acid sequence in the polypeptide chain and therefore, they can be separated by adjusting the pH of a solution. When the pH is adjusted to the isoelectric point of a particular protein it gets precipitated leaving the other proteins in the solution, which could be separated using centrifugation (McClements, 2013; Garba and Kaur, 2014). Isoelectric precipitation (IEP) could efficiently help in the recovery of high quality fish protein isolate but there have been no successful food products launched in the market other than nutraceutical products from fish processing waste that can be used for human consumption (Tahergorabi *et al.*, 2015).

Fish protein isolate (FPI) is a product which is having higher protein content and is lower in carbohydrates and fat. According to Garba and Kaur (2014), protein isolates are refined form of protein with higher digestibility and also having high functional properties like gelation, foaming ability and emulsification. FPI can be prepared from fish muscle and also from surimi processing waste and is not generally consumed directly, but used as raw material for production of value added products (Garba and Kaur, 2014). The use of fish species with higher content of dark/red muscle and fat has low grade protein gels, colour problems and lipid oxidation and hence species with white flesh and low fat content are generally preferred for surimi manufacturing (Park and Lanier, 2000). Shaviklo (2006) studied the quality of fish protein isolate based on the Codex Code of Practice for frozen surimi.

In India, there are eight surimi plants which are registered under Export Inspection Agency (MPEDA, 2016). Surimi is a value added product which gains more value in the international markets. The main waste components generated during surimi processing are head, skin, fin, tail, bone and frames. Japanese Threadfin bream *Nemipterus japonicus,* is mainly used as the raw material for surimi production in India, because of its abundance, low cost, white meat and low fat content (Singh and Balange, 2005). At present, waste generated from surimi processing units are used for the production of fish meal or directly used in animal feeds but more frequently is simply discarded. IEP process facilitates selective, pH induced water solubility of proteins which retain both functional and nutritional properties (Lee *et al.,* 2016) along with concurrent separation of lipids and removal of materials not intended for human consumption (such as scales and bones). The present study attempted to utilise isoelectric precipitation technique to recover fish protein isolate from *N. japonicus* processing waste.

Materials and methods

Raw material

N. japonicus processing waste comprising head, viscera and frames were obtained from Chambakkara and Thoppumpady fish markets, Kochi, Kerala. Samples were transported in ice in an insulated box to the laboratory with in 2 h. The samples were weighed and packed in polyethylene bags and stored at -18°C until further use.

Extraction of fish protein isolate by isoelectric precipitation

Partially frozen threadfin bream processing waste were ground into a paste and mixed with cold distilled water at different ratios (1:3, 1:5 and 1:9) of fishwaste:water (w/v) in five replications separately and homogenised using a laboratory homogeniser (IKA Ultra turrax, model no: T18 digit) at a speed of 1500 rpm for 60 s to form a uniform mixture. The homogenate was poured into a glass beaker maintained at chilled temperature (4^0C) which was further used for solubilisation and precipitation of protein. The details of experimental treatments are provided in Table 1.

Table 1. Experimental treatments

Treatments	pH	Fish waste : Water ratio
T1	10	1:3
T2	11	1:3
T3	12	1:3
T4	13	1:3
T5	14	1:3
T6	10	1:5
T7	11	1:5
T8	12	1:5
T9	13	1:5
T10	14	1:5
T11	10	1:9
T12	11	1:9
T13	12	1:9
T14	13	1:9
T15	14	1:9

pH of the homogenate was adjusted to 10, 11, 12, 13 and 14 respectively with 2N NaOH by slow and continuous stirring using a glass rod and pH was monitored using a pH meter (EUTECH, model no: 510). The solubilisation reaction lasted for 25 min during which the OH^- ions gets accumulated around the protein and induces protein-protein electrostatic repulsion leading to solubilisation of more protein in water (Undeland *et al.,* 2003; Kristinsson *et al.,* 2005). The solubilised solution was then poured into 50 ml tubes and centrifuged at 10,000 rpm at 4°C for 10 min in a refrigerated centrifuge (REMI, model no. C-24BL). The first centrifugation gave three separate layers such as (i) top layer containing thin layer of fat; (ii) middle layer containing solubilised protein and (iii) bottom layer containing insoluble materials likes bones, scales and skins (Fig. 1). The supernatant solution was poured through a Whattman no. 2 filter paper, to remove fat. pH of the solubilised protein solution was then adjusted to 5.1-5.6 (Xiong, 1997; Choing and Yeung, 2005; Zhong *et al.,* 2015) using 2N HCl by slow and continuous stirring for the best precipitation of protein. The precipitation reaction lasted for 10 min and then the solution was poured into 50 ml tubes and centrifuged at 10,000 rpm at 4°C for 10 min using refrigerated centrifuge to separate FPI.

Analysis of fish processing waste and recovered fish protein isolate

Proximate composition

The proximate composition of fish processing waste and the extracted fish protein isolate (FPI) obtained from the different treatments was analysed in triplicates following AOAC (2000). Moisture was determined by oven drying to constant weight at 105°C for 24 h. Crude protein content was determined using a Kjeltec Auto analyser

(Nitrogen×6.25; Vapodest-50, Gerhardt, Germany). Crude lipid was determined by Soxhlet method using petroleum ether (boiling point 60-80°C) as solvent. The ash content was determined by combustion of samples at 550°C for 4 h in a muffle furnace.

Protein recovery

Protein recovery was calculated based on the weight of recovered protein divided by the weight of fish processing waste. After acidic/alkaline treatment for IEP process, the weight of recovered protein was recorded and its moisture content was also measured (Kim *et al.,* 2003).

$$\text{Protein recovery (\%)} = \frac{\text{Wt. of recovered protein at \% moisture}}{\text{Wt. of fish processing waste}} \times 100$$

Yield of fish protein isolate (FPI)

The yield of FPI was calculated by dividing the weight of recovered FPI by the weight of fish processing waste used for homogenisation.

$$\text{FPI Yeild (\%)} = \frac{\text{Wt. of FPI}}{\text{Wt. of fish processing waste}} \times 100$$

Statistical analysis

All analyses were performed in triplicates and data were analysed by One-way ANOVA using SPSS software (SPSS Inc., Chicago, USA). Duncan's multiple comparison tests was used to determine the differences between the treatment means. Results were considered statistically significant at $p<0.05$.

Results and discussion

Proximate composition

The proximate composition of the fish processing waste and the extracted FPI obtained from the different treatments are presented in Table 2 and 3 respectively. Threadfin bream processing waste contained 12.71±0.46% of crude protein, 8.45±0.23% crude fat and 3.23±0.62% ash, on wet weight basis. Protein content of extracted isolate showed an increasing trend from 6.39±0.93% in T1 and highest (19.63±0.52%) in T14 (Table 2). The lowest lipid content was recorded in T5 (1.03±0.13%) and highest in T1 (1.61±0.65%). Ash content of the recovered FPI also showed a declining trend from 0.97±0.021% in T1 to 0.79±0.02% in T15.

Effect of pH and water ratio on the yield of FPI

The second centrifugation step gave two separate layers consisting of supernatant layer as clear water and bottom layer which is the FPI (Fig. 2). The final product obtained after the process is shown in Fig. 3. The protein recovery of FPI at sample to water ratio of 1:3 increased from 6.41±0.91 to 14.67±0.48% when the pH shifted from 10 to 14. Similar increase was also obtained for sample to water ratio of 1:5 with lowest yield of 13.89±0.6% for pH 10 and highest 17.17±0.49% at pH 14. In the case of 1:9 ratio, comparatively higher FPI yield was obtained for all pH conditions ($p<0.05$) with highest yield of 19.63±0.53% recorded at pH 13 (Table 3). The results of yield percentage of FPI from the extracted protein was lowest % in 1:3 sample to water ratio at pH 10 (36.34±0.11) ($p<0.05$) and highest in sample to water ratio of 1:9 at pH 13 (84.13±0.11) (Table 4).

Proximate composition of protein isolate is greatly affected by the pH and sample to water ratio. Proximate composition of the raw material (*N. japonicus* processing waste) used in the isolation process of FPI had crude protein content of 12.71±0.46%; with fat and ash content of 8.45±0.23 and 3.23±0.62% respectively. The sample extracted from the treatment T14 showed the highest crude

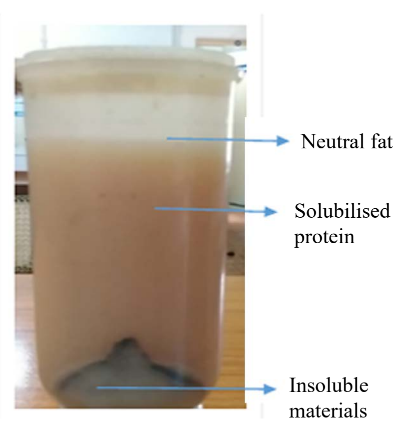

Fig. 1. Three separate layers formed after first centrifugation of the solubilised protein from the fish processing waste

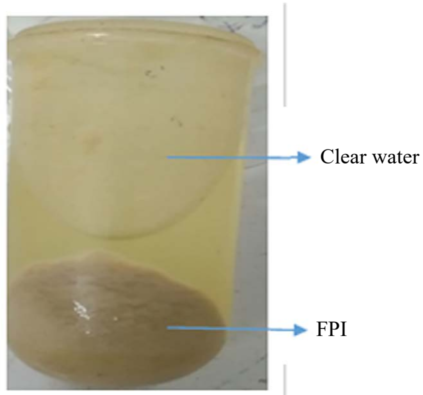

Fig. 2. Two separate layers comprising of supernatant layer as clear water and bottom layer of FPI after the second centrifugation step

Fig. 3. Fish protein isolate obtained after isoelectric precipitation of solubilised protein

Table 2. Proximate composition of threadfin bream processingwaste and fish protein isolate

Sample	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Thread fin bream processing waste	75.17 ± 1.16	12.71 ± 0.46	8.45 ± 0.23	3.23 ± 0.62
Fish protein isolate				
T1	76.22 ± 0.07	6.39 ± 0.93	1.61 ± 0.65	0.97 ± 0.02
T2	76.87 ± 0.66	8.61 ± 0.51	1.39 ± 0.05	0.95 ± 0.01
T3	77.90 ± 0.05	10.49 ± 0.48	1.32 ± 0.58	0.92 ± 0.01
T4	78.69 ± 0.14	14.22 ± 0.47	1.17 ± 0.05	0.93 ± 0.01
T5	78.44 ± 0.88	14.66 ± 0.47	1.03 ± 0.12	0.95 ± 0.01
T6	75.34 ± 0.60	13.89 ± 0.60	1.51 ± 0.55	0.91 ± 0.16
T7	76.05 ± 0.56	14.30 ± 1.13	1.42 ± 0.04	0.91 ± 0.01
T8	76.85 ± 0.07	15.75 ± 0.51	1.41 ± 0.03	0.89 ± 0.01
T9	78.04 ± 0.08	16.63 ± 0.48	1.34 ± 0.11	0.86 ± 0.02
T10	78.07 ± 0.06	17.17 ± 0.49	1.19 ± 0.05	0.87 ± 0.23
T11	75.28 ± 0.03	14.55 ± 1.50	1.45 ± 0.09	0.90 ± 0.01
T12	74.97 ± 0.02	17.35 ± 0.97	1.40 ± 0.08	0.81 ± 0.01
T13	75.98 ± 0.07	17.88 ± 0.53	1.23 ± 0.52	0.89 ± 0.03
T14	76.48 ± 0.06	19.63 ± 0.52	1.21 ± 0.02	0.92 ± 0.03
T15	76.69 ± 0.05	19.45 ± 0.51	1.06 ± 0.07	0.79 ± 0.02

Values are Mean±SD from triplicate observations.
Concentrations are on wet weight basis

Table 3. Protein recovery (%) of FPI extracted from threadfin bream processing waste

Sample:Water ratio	pH				
	10	11	12	13	14
1:3	6.41±0.91^{a}	8.61±0.51^{b}	10.49±0.48^{c}	14.22±0.47^{d}	14.67±0.48^{d}
1:5	13.89±0.60^{a}	14.31±1.13^{a}	15.75±0.52^{b}	16.63±0.48^{bc}	17.17±0.49^{c}
1:9	14.57±1.50^{a}	17.35±0.97^{b}	17.89±0.53^{bc}	19.63±0.53^{d}	19.45±0.51^{cd}

Data are presented as Mean±SD of three independent replications. Values with different superscripts in the same row differ significantly ($p<0.05$)

Table 4. Yield % of FPI from the extracted total protein

Sample:Water ratio	pH				
	10	11	12	13	14
1:3	36.34±0.11^{a}	47.50±0.16^{b}	58.43±0.22^{c}	70.51±0.23^{e}	63.03±0.11^{d}
1:5	60.05±0.27^{b}	66.08±0.21^{c}	70.04±0.17^{d}	73.04±0.16^{e}	59.13±0.13^{a}
1:9	63.15±0.31^{b}	65.41±0.22^{c}	72.16±0.16^{d}	84.13±0.11^{e}	60.12±0.12^{a}

Data are presented as Mean±SD of three independent replications. Values with different superscripts in the same row differ significantly ($p<0.05$)

protein of 19.63±0.52% on wet weight basis and 83.46% on dry weight basis. A similar result on crude protein content of FPI was also reported for tilapia fillet waste extracted at pH 12 with highest crude protein of 19.19%, followed by acid extraction at pH 2 yielding 16.81% (Chomnawang and Yongsawatdigul, 2013). Protein isolate extraction from silver carp processing waste using acid and alkali aided methods yielded 46.56 and 70.92% (on dry weight basis) respectively which indicated that extreme pH treatment resulted in more protein recovery (Zhong *et al.*, 2016). The protein content of protein isolate prepared from yellowfin tuna roe by IEP process were in the range of 11.6-14.1% (on wet weight basis) (Lee *et al.*, 2016). The IEP technique efficiently removed fat from the protein isolate and the recovered fish protein isolate contained lipid and ash in the range of 1.03-1.61 and 0.79-0.97%, respectively as compared with the raw material used for FPI which had 8.45 and 3.23% of lipid and ash respectively. Mostly ash content is higher in the bones, head and fins. Results from the study imply that most of the unsolubilised matter was separated during the IEP process. Lipid and ash content can be used as indicators of the level of removal of impurities from the recovered proteins. A similar result reported on lipid content of FPI from silver carp as 4.3 to 8.3% (on dry weight basis) using alkali aided extraction which resulted in greatest fat removal (Zhong *et al.*, 2016). According to Chomnawang and Yongsawatdigul (2013), alkali pH-shift process is an efficient means to remove fat from tilapia fillet byproduct. Liang and Hultin (2003) also found reductions in the total fat content from 10.8 to 0.9% in coarsely ground deboned turkey meat after being subjected to an alkali-aided process. Similar results were also reported by Kristinsson and Demir (2003) who revealed that an alkali pH-shift process reduced fat to a greater extent than did the acid counterpart.

During the extraction process of FPI from the threadfin bream processing waste, treatment T14 showed the highest yield. The pH and sample to water ratio showed a significant effect in the FPI yield ($p<0.05$). In the pH-shift method, the protein source is first mixed with water to obtain a medium to solubilise the proteins *i.e.,* homogenisation. The proteins are homogenised to increase the interaction surface between proteins and water (Hultin and Kelleher, 1999). Thus water has a significant role in protein solubilisation and protein solubility in an aqueous solution is dependent on pH.

Maximum yield of FPI was obtained when sample to water ratio was 1:9 and pH was 13 in this study. The results corroborates the findings of Chomnawang and Yongsawatdigul (2013) that in the water ratio of 1:9, highest solubility of protein was observed in tilapia filleting waste and lowest occurred at the ratio 1:3. According to Yasothai and Giriprasad (2015), homogenisation is essential to maintain low viscosity, which is necessary to separate insoluble material from soluble proteins. Viscosity of a protein solution may be affected by factors like protein concentration, pH, salt and raw material which can in turn affect the size, shape, flexibility and hydration of the proteins (Ingadottir, 2004). Batista (1999) suggested that the use of a high ratio of solvent during pH-shift process resulted in a very dilute extract that was inefficient to recover protein. But, low ratio gave more viscosity, making it very difficult to centrifuge and separate solid particles. Hultin *et al*. (2005) suggested that protein extraction in pH-shift method can be accomplished in 5-10 volumes of water with added alkali or acid.

Maximum protein solubility in the present study was obtained at the pH 13. At pH 5.2, a large amount of precipitates was formed after centrifugation due to the low electrostatic repulsion between protein molecules. Therefore, pH 5.2 was determined as the isoelectric point of threadfin bream processing waste proteins. To obtain high yield of good quality protein, it is necessary to solubilise a large portion of the proteins (Undeland *et al*., 2002). Yeung and Soo (2005) investigated the solubility of croaker and Jack mackerel proteins using acid/alkali process and showed higher recovery of fish proteins at the pH 2.0 and 11 and the lowest solubility obtained at pH 4.5 and 5.5, suggesting that pH 5.5 is the isoelectric point where proteins have zero net charge in solution, resulting in minimum solubility and maximum precipitation. Once the pH shifted away to either acid or alkaline direction from the isoelectric point, electrostatic repulsion between molecules increases, resulting in high protein solubility. Xiong (1997); Choi and Kim (2005) and Zhong *et al,* (2015) conducted similar studies with pH adjusted to a range of 5.1-5.6 to obtain maximum protein precipitation. Using the acid and alkaline processes, Undeland *et al*. (2002) got protein yields of 74±4.8 and 68±4.4% at pH 2.7 and 10.8, respectively, from white muscle of herring. Kristinsson and Ingadottir (2006) investigated protein yields from tilapia using acid and alkali aided processes and reported 56 to 61% yield with acid aided process (pH 2.3-2.9) and from 61 to 68% with alkali aided process (pH 10.8-11.4). Kim *et al.* (2003) evaluated acid and alkali aided processes on Pacific whiting at pH 2, 3, 10.5, 11 and 12 and used a 1:10 fish to water ratio. The highest protein yield was found to be 70% at pH 12 and the lowest protein yield was 60% at pH 10.5. Batista (1999) reportred protein yields of 80.6 and 62.9% in isoelectric precipitation of alkali aided process of hake and monkfish, respectively. Batista *et al.* (2003) later investigated protein recovery from sardine (*Sardina pilchardus*) and blue whiting mince. They used similar acid and alkaline processes and obtained 73 and 77% protein recovery, respectively, from sardine mince and 53.6 and 49.1%, respectively, from blue whiting mince. The present study concluded that IEP technique is highly effective to extract up to 84% of FPI from threadfin bream processing waste using sample to water ratio of 1:9 and at solubilisation pH of 13 and precipitation pH of 5.2.

Isoelectric precipitation (IEP) is a promising method for the preparation of fish protein isolate (FPI) from seafood processing waste. This technology can be adopted by the fish processing plants as an effective strategy for waste management. Adoption of this method by the seafood processors can lead to the reduction in solid fish waste, additional profit through FPI extraction and more significantly it would help to reduce environmental contamination.

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