Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(4): 710-719

Received: 19-05-2020
Accepted: 21-06-2020

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Optimization of the process for recuperating proteins from Pacu processing waste: Quantification and characterization of isolates

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Abstract
Pacu processing waste was used to recuperate proteins from it using pH shift process, followed by the quantification and detailed characterization of recovered Pacu protein isolates. Maximum protein solubility was recorded at pH 2.0 (16.13 ± 0.30 mg/ml) and pH 12.0 (14.67 ± 0.26 mg/ml), with a total process recovery of 63.49 ± 0.36 and 53.53 ± 0.21%, respectively. Process variables showed significant effect of protein solubility and recovery yields (p<0.05). Maximum solubility was reported with the extraction time of 60 min at 50ºC, weight to extractant volume ratio of 1:3, centrifugation speed of 8000 rpm and continuous stirring condition. Alkaline processed isolate had higher protein content (22.87 ± 0.74%) than acidic processed isolates (21.01 ± 0.88%). The protein isolate obtained through acidic/alkaline aided processing had a reduced level of total lipid, myoglobin and pigments as compared to the raw material. Foaming capacity was higher in acidic aided protein isolate than in alkaline based protein isolate, whereas foaming stability, water holding capacity and emulsion capacity were higher in alkaline processed isolate than acid processed isolates (p<0.05). Proteins recovered from Pacu fish by acid/alkali solubilisation method showed good yields and functional properties. The alkaline aided protein isolation was proved to be better in terms of protein yield as well as quality of isolates. The recovered proteins showed higher protein concentrate, gelling and texture properties as well as good amino acid profile.

Keywords: Pacu, protein, mince, alkaline, isolates and gel

Introduction
Fish is human's one of the most prominent origin of high-quality protein, which also contain excellent amounts of essential fatty acids, vitamins and minerals. The annual world catch of wild fish and invertebrates has stabilized around 178.5 million tons feeding to 7.6 billion humans in which 156.4 million tons is directed towards human food, while 22.2 million tons used for non-food uses like pharmaceuticals, bait, ensilage, animal feed etc. [9]. Pacu (*Piaractus brachypomus*) (Cuvier, 1818) is one of the exotic important cultured fish, belongs to the family *Characidae*. The production of the fish varies from farm to farm and overall production in the country is estimated to cross 0.2 million tonnes/ha/year [1]. Processing of Pacu fish generates huge amount of waste, which occasionally makes up to 50% of the total body weight, polluting local water bodies and lands. During the filleting considerable amount of meat will be remained as leftover with the fillet frames. This leftover meat contains valuable components which can be used for edible and nonedible purposes [40]. Hence, it is necessary to optimize the process and evaluate the quality of protein isolates from various raw materials and processes. [39, 40]. Many attempts have been made both in academia and industry to retrieve or isolate proteins from fish by-products and underutilised fish species [24]. New technology has been improved to enhance fish-based protein food for individual consumption. According to Thorkelsson, 2008 [48] this innovation which is claimed the acid and alkali processes or pH-shift method has been detailed and promoted by many researchers [3, 16, 17]. No report has been published or due to scanty information on the recovery and application of fish protein isolate from Pacu fish, this research concentrates on optimization and characterisation of protein isolates recovered from this exotic fish using pH shift method.

Materials and Methods

Raw materials

Pacu fish (*Piaractus brachypomus*) purchased in fresh condition from local fish market, Mangalore and brought to the testing laboratory Department of Fish Processing Technology, COF, KVAFSU, Mangalore under iced condition (1:1) in insulated boxes, stored at 4 °C till further usage (within 24 h). The fish fillet was prepared manually and made into fine paste using a meat mincer followed by pH shift processing. All steps were performed at temperature below 5 °C to avoid quality losses.

Preparation of homogenate

Minced meat was mixed with cold deionized (DI) water (4 °C, 1:9, W:W) and subjected to homogenization for 60s (2 × 30s) with a homogenizer (Ultra-Turrax, T 25, Janke and Kunkel GMBH and Co., Staufen, Germany). The homogenate was adjusted to the desirable pH using 2 M HCl or 2 M NaOH with continuous stirring.

Protein solubility

Solubility curve

Protein solubility curve was constructed by adjusting the pH of the homogenate between pH 3.0 to 11.0 with 1.0 unit interval. Protein solubility at each pH was calculated to construct solubility curve [43].

Protein measurements

The total protein content of homogenate and supernatant was measured using Biuret method [52]. The cloudiness due to lipids in the solution was lowered by adding 10% deoxycholic acid. Absorbance was read at 540 nm using a T90+ UV/VIS spectrophotometer (PG Instruments Ltd., India), and protein concentrations were measured from a standard curve developed using bovine serum albumin (BSA) at a concentration of 1–10 mg/ml.

Protein recovery calculation

Protein recovery was expressed as theoretical and actual recovery (%). Both theoretical and actual recoveries were classified into (a) recovery after 1st centrifugation, (b) recovery after 2nd centrifugation, and (c) total process recovery. Theoretical recoveries were calculated using the formulas presented below:

Protein recovered after 1st centrifugation: $\% \ Protein = \frac{B}{A} * 100$

Protein recovered after 2nd centrifugation: $\% \ Protein = 100 - [\frac{C}{B} * 100]$

Protein recovered through the whole process: $\% \ Protein = \left[B - \frac{C}{A}\right] * \ 100$

Where, 'A' is the soluble protein in initial homogenate, 'B' is the soluble protein in supernatant after first centrifugation, and 'C' is the soluble protein in supernatant after second centrifugation. Equations for actual recovery were exact to theoretical recovery, except the weight of supernatant and homogenate, which were multiplied with the protein concentration.

Preparation of protein isolates

The ground muscle was stirred with cold DI water (1:9, w/w), homogenized for 60s (2 × 30s) with a Waring blender (Waring Products Division, New Hartford, CT, USA) at 40% electrical production and flooded (to avoid foaming) into a plastic beaker set on ice. To solubilize the proteins, the homogenates were adjusted to pHs 2.0 and 13.0 (~10 min), employing 2 M HCl or 2 M NaOH. Then, the homogenate was centrifuged at 10,000×g for 20 min. The top layer and debris were discarded and the supernatant was taken to isoelectric precipitation by adjusting the pH to 5.5, followed by centrifugation at 10,000×g for 20 min. The resulting sediment (isolate) was packed in a zip-lock bag and kept on ice in a cold room at 4 °C overnight.

Proximate composition

The proximate composition (%) such as moisture, protein, ash, and fat content of the raw meat and Pacu protein isolate, were analysed by the standard protocol described by AOAC [2].

Color analysis

Color characteristics of the homogenate and isolates were assessed using HunterLab (Reston, VA, USA) as defined by [6]. Lightness (L*), redness/greenness (a*) and yellowness/blueness (b*) were observed, and whiteness was determined using the formula.

$$Whiteness = 100 - [(100 - L)^2 + a^2 + b^2]^{1/2}$$

Lipid content

Total lipids (Dry weight basis) in the homogenate and protein isolates were determined using soxhlet apparatus as per the method described in AOAC [2]:

% Fat (Dry weight) = (g of fat in sample/g of dried fish sample) x 100

Total pigment content

Sample (1 g) was mixed with acetone acid (9 ml) containing 90% acetone, 8% deionized water and 2% HCl [35]. After careful stirring, the mixture was maintained at room temperature for 1 h. Absorbance of the filtered solution was noted at 640 nm using acetone as a blank. A ratio of 680 and specimen weight was used to calculate total pigment concent (haematin).

Total pigment content (ppm) = $A640 \times 680$

Total myoglobin content and its removal

Total myoglobin content of homogenate and isolate was determined [5] by taking 2 g of sample and adding 20 ml of 40 mM, pH 6.8 phosphate buffer, followed by homogenization (3000 rpm for 30 min at 4°C) and filtration using Whatman no. 1 paper. Supernatant was added with 0.2 ml of 1% (w/v) sodium dithionite followed by absorbance measurement at 555 nm using phosphate buffer (pH 6.8) as a blank. Myoglobin content was determined as below:

$$Myoglobin\ content\ (mg/kg) = (A \times 16.111 \times F \times Ws \times 7.6) \times 100$$

Where,
A = absorbance,
F = dilution factor,
Ws = weight of sample in g,
7.6 = millimolar extinction coefficient,
16.111 = molecular weight

Foaming properties

About 1 g of protein sample was put in 50 ml distilled water. The sample was suspended at 60 °C, and foam was produced by homogenizing at 9,000 rpm for 5 min using Ultra Turrax homogenizer (Ultra-Turrax, T 25, Janke and Kunkel GMBH and Co. KG). The homogenized solution was drained into a measuring cylinder of 250 ml capacity. The foam formation capacity was measured as the volume ratio of foam to the initial liquid volume and showed as percentage. The foam stability was determined as the ratio of the initial volume of foam to the volume of foam after 30 min [37].

Emulsification capacity

Soybean oil (5 ml) was mixed with fish protein isolate (FPI) solution (1%) and mixed well [11]. It was subjected to high speed homogenization (17000 rpm) for 2 min followed by centrifugation at 1096×g for 15 min. The three fractions appeared in the tube after the centrifugation was calculated independently and the emulsification capacity (EC) was measured applying the accompanying formula.

$$Emulsification\ capacity\ (mL/\ 100mL) = \frac{Emulsified\ oil\ (mL)}{FPI} \times 1$$

Water holding capacity and oil holding capacity

For WHC (water holding capacity) measurement, 10 ml of deionized water was mixed with 1 g sample and was vortexed for 30s followed by centrifugation at 875×g for 25 min. Supernatant volume was noted and the WHC was expressed as the millilitres of water absorbed per gram of sample. For OHC measurement, soybean oil (10 ml) was added to sample (1 g) supported by stirring and vortexing for 30s. This was followed by centrifugation (875×g) for 25 min. After the centrifugation, the free oil was discarded and OHC was determined using the weight differences [11].

Gel preparation

For producing protein gels, isolates were cut into 1 cm thick slices and ground using a laboratory mincer [25]. Protein isolates were adjusted to a uniform moisture content (80%) and were mixed with 2.5% salt (HiMedia, Mumbai, India). The mixture was exposed to fine mixing, chopping followed by stuffing into cellulose casings of 2.5 cm dia (Dr Froeb (I) Pvt. Ltd, Noida, India). Stuffed casings were closed using a sealer followed by incubation for 30 min at 40° C. The sausages were heat treated for 20 min at 90° C accompanied by cooling in chilled potable water for 30 min. All the sausages were incubated overnight at 4° C for further analysis.

Gel quality analysis

Folding test and gel strength

Method described by Kudo *et al.*, 1973 [28] was used for performing folding test of the protein isolate gels. Gel strength of protein isolate gels were measured as outlined by Reddy, 2016 [36], using a texture analyzer (TA-XT plus, Stable Micro Systems, Godalming, UK). Specimens (2.5 cm × 2.5 cm) were compressed to a distance of 10 mm using a spherical probe (5 mm) with 20 g target force, 1 mm s^{-1} test speed and 2 mm s^{-1} pre- and post-speed. Obtained data was calculated using TA-XT plus program.

Expressible moisture

Gel sample of 3 mm thickness was kept between five layers of Whatman filter papers and compressed by a metal standard weight of 3000 g for 1 min [10]. The expressible moisture content was calculated using;

$$Expressible\ moisture\ (g/100g) = \left[\frac{prepressed\ wt.of\ gel - after\ pressed\ wt.of\ gel}{prepressed\ wt.of\ gel}\right] \times 100$$

Amino acid profiling

Amino acid profiling of homogenate and protein isolate of Pacu was done using reverse phase HPLC on Water Picotag TM amino acids analysis system, after hydrolyzing the protein with 6 N HCl to yield free amino acid [4]. The amino acid content of Pacu protein, comprising the leading substance, was measured and showed as g/100 g protein.

Statistics analysis

All the results were presented in triplicate unless otherwise stated. All the readings were tested using SPSS (Version 20) software. *t*-test was adopted to find significant differences ($P<0.05$) between the treatment attributes and the values were expressed as mean ± standard deviation.

Results and discussion

Effect of pH on Pacu protein solubility and recovery yield

Solubility of Pacu proteins at various pH's during acidic and alkaline solubilization and precipitation is given in Table 1. Maximum protein solubility was recorded at pH 2.0 (16.13 ± 0.30) and pH 12.0 (14.67 ± 0.26 mg/ml), respectively ($P<0.005$). However, the protein had lowest solubility at pH 5.5, which could be the reason related to the isoelectric point of that protein. Above and below this pH spectrum, protein turns more positive and negative charge, therefore raising the solubilization by electrostatic repulsion [15, 23]. The rapid increase in solubility between pH 2.5 and 7.0 compared to the steady increase in solubility between pH 7.0 and 11.0 might be because of more ionizable groups with pKa values between pH 2.5 and 7.0 [49]. Many studies have reported that maximum solubility of fish proteins would be at pH 2.0 to 3.0 from acidic side and pH 11.0 to 13.0 from alkaline side, with least solubility at pH 5.0 to 6.0. Some findings revealed subtle transition in solubility between pH 3.5 and 4.0 and pH between 9.5 and 11.0 [45]. The importance of any extraction process depends on process yields and recovery rate [44]. Theoretical recovery was found to be more than actual recovery for all the pH's studies.

Table 1: Protein recovery (g/100 g) from Pacu deboning waste after 1st centrifugation, using pH shift method

ID	Protein solubility (mg/ml	Protein recovery after I centrifugation (%)		
		(A) Theoretical recovery (%)	(B) Actual recovery (%)	*P* Value (A, B)
pH 1	12.47 ± 0.33^{e}	38.14 ± 0.84^{d}	32.78 ± 0.45^{d}	<0.001
pH 2	16.13 ± 0.30^{a}	52.51 ± 0.34^{a}	48.67 ± 0.34^{a}	<0.001
pH 3	13.10 ± 0.11^{cde}	44.07 ± 0.73^{b}	35.38 ± 0.52^{c}	<0.001
pH 4	10.26 ± 0.37^{f}	28.61 ± 0.31^{h}	24.72 ± 0.25^{g}	<0.001
pH 5	04.35 ± 0.38^{i}	24.45 ± 0.51^{i}	20.11 ± 0.45^{h}	<0.001
pH 5.5	04.41 ± 0.21^{i}	18.80 ± 0.56^{k}	15.88 ± 0.59^{i}	<0.003
pH 6	06.45 ± 0.20^{h}	20.42 ± 0.25^{j}	16.76 ± 0.38^{i}	<0.001
pH 7	07.62 ± 0.50^{g}	23.01 ± 0.72^{i}	19.64 ± 0.71^{h}	<0.004
pH 8	08.46 ± 0.42^{g}	30.91 ± 0.58^{g}	26.97 ± 0.50^{f}	<0.001
pH 9	09.62 ± 0.53^{f}	32.50 ± 0.44^{f}	27.82 ± 0.98^{f}	<0.002
pH 10	12.46 ± 0.29^{de}	34.60 ± 0.42^{e}	29.91 ± 0.82^{e}	<0.001
pH 11	13.93 ± 0.51^{bc}	39.77 ± 0.55^{c}	34.58 ± 0.44^{c}	<0.001
pH 12	14.67 ± 0.26^{b}	42.66 ± 0.46^{b}	39.16 ± 0.70^{b}	<0.002
pH 13	13.51 ± 0.43^{cd}	34.69 ± 0.22^{e}	28.26 ± 0.61^{ef}	<0.001
P Value	<0.001	<0.001	<0.001	

Note: Different superscripts in small letters (a, b, c) indicate significant difference (p<0.05) amongst column. Value are expressed as mean ± SD (n=3)

Protein recovery yields were 48.67 ± 0.34% and 39.16 ± 0.70% after first centrifugation and 57.40 ± 0.31 and 44.58 ± 0.26% after second centrifugation in acidic and alkaline solubilization, respectively (Table 2). The total process recovery was 63.49 ± 0.36 (acidic) and 53.53 ± 0.21% (alkaline). Several factors regulate the recoveries at acid and alkaline pH, such as; solubility, size of the debris formed during the centrifugation, and solubility at isoelectric pH, etc. Among these, the solubility at different pH is the major cause that influences recoveries [45]. Some investigations showed that pH shift processing give higher protein recoveries compared to the conventional surimi processing [26, 35, 19], while some reports highlights acid-assisted process give greater protein recoveries than alkaline-assisted process [35]. The reason for high protein recovery in pH shift process compared with protein recovery using conventional surimi processing was described by researchers in many research studies [8, 47].

Table 2: Recovery and process yields of protein from Pacu fillet frames during solubilization at pH 2.0 and 12.0.

Recovery and Yields	Acid processed isolate (pH 2.00)	Alkaline processed isolate (pH 12.0)	*P* value
Theoretical recovery after first centrifugation (%)	52.51 ± 0.34^{c}	42.66 ± 0.46^{c}	<0.001
Actual recovery after first centrifugation (%)	48.67 ± 0.34^{d}	39.16 ± 0.70^{d}	<0.001
Theoretical recovery after second centrifugation (%)	53.43 ± 0.50^{c}	45.38 ± 0.49^{b}	<0.001
Actual recovery after second centrifugation (%)	57.40 ± 0.31^{b}	44.58 ± 0.26^{b}	<0.001
Total recovery (%)	63.49 ± 0.36^{a}	53.53 ± 0.21^{a}	<0.001
P value	<0.001	<0.001	

Note: Different superscripts in small letters (a, b, c) indicate significant difference (p<0.05) amongst column. Value are expressed as mean ± SD (n=3)

Effect of process variables on protein solubility and recovery yields

Process variables such as extraction time, temperature, homogenate to water ratio, centrifugation speed and stirring time caused significant differences (p<0.005) in protein yields (Table 3). Maximum solubility was reported with the extraction time of 60 min (22.50 ± 0.36) and recovery yields were steadily improved from 5 to 60 min followed by a gradual decrease from 60 to 120 min. Theoretical recovery was found to be better than actual recovery for all the extraction times tested. Uneven dispersion of non-protein substances in the homogenate used for the recovery at various extraction times might be the reason behind the variations in solubility and recovery. The duration of extraction time affected the solubility of hake and monk fish proteins considerably [3]. They found an increasing solubilization of protein with increasing extraction time. The present findings are also in agreement with the previous reports [21, 31, 3, 46, 42].

The extraction temperature also had significant effect on protein solubility (p<0.005), which increased from 11.48 ± 0.39 to 19.47 ± 0.38 mg/ml with increase in temperature from 4ºC to 50ºC. These findings are supported by the findings of previous workers [43, 42, 39] during their work on pangus and rohu protein isolates, while the trend was not observed while working with the proteins of flounder and squid [31, 21].

With respect to homogenate weight to solvent ratio, the minimum protein solubility (12.44 ± 0.42 mg/ml) was noted at 1:20 (W: V) ratio and maximum was at 1:3 ratio (27.71 ± 0.57 mg/ml). Similar pattern was observed by [39, 45, 40, 3] with pangas and rohu fish proteins and fish waste. Montecalvo, 1984 and Batista, 1999 [31, 3] reported that the use of significant ratios of extractant and ground homogenate resulted in certain dilute solutions of substantial volumes causing trouble in handling, while low ratios gave high concentrated protein solutions causing trouble in recovery due to excessive viscosity and gelation. Selecting the exact ratio of tissue to extractant becomes a crucial factor for better yields. A ratio of 1:10 found to be satisfactory for yield, convenience and handling, while a ratio of 1:7 was also satisfactory [31]. In this study, a ratio of 1:6 (w:v) found to be more convenient for efficient protein yields and handling. Kahn, 1974 [21] reported that tissue to extraction ratio of 1:10 gave best results during the study of squid protein isolates.

Protein solubility was maximum with centrifugation speed of 8000 rpm (23.23 ± 0.59 mg/ml) and minimum at 2000 rpm (17.83 ± 0.69 mg/ml). The reason for low solubility at lower centrifugation speed could be due to not generating an ample force that separate solubilized proteins from insolubilized

matter and lipids thus by causing a fluid-gel like sediment resulting in low recoveries. Kain, 2009 [22] reported identical results during their work on peanut protein isolates. Surasani, 2016 [46] also used the same method while recovering proteins from rohu fish waste and the findings were in support with the current findings.

The time of stirring also makes more or less impact on the protein solubility and its recovery values ranged from minimum to maximum as 14.40 ± 0.48 to 20.44 ± 0.40 mg/ml during without stirring and with stirring condition, respectively. However, theoretical protein recovery was reported to have significant change (p<0.005) compared to actual protein recovery. The present results indicated that time of stirring slightly improved protein recovery rate and protein solubility statistically but differences were very less. Shen, 1975 [38] during his studies on soy protein isolation used a moderate shaking at 11000 rpm for 2 h to blend and found that increasing blending speed had a noticeable effect on protein solubility. During stirring there is a continuous mixing, inducing an enhanced interaction between protein and water molecules, thus by solubilizing more proteins within the time.

Table 3: Effect of process variables on solubility of proteins from Pacu fillet frame during alkaline solubilization

Process variables	Protein solubility (mg/ml	Recovery (%)		
		Theoretical	Actual	*P* Value
Extraction time (Minutes)				
5	19.70 ± 0.42^{c}	69.36 ± 0.33^{d}	65.57 ± 0.40^{d}	<0.001
30	21.50 ± 0.48^{b}	72.51 ± 0.41^{b}	70.45 ± 0.29^{b}	<0.002
60	22.50 ± 0.36^{a}	75.59 ± 0.33^{a}	72.47 ± 0.54^{a}	<0.001
90	20.80 ± 0.24^{b}	70.57 ± 0.34^{c}	67.60 ± 0.40^{c}	<0.001
120	19.61 ± 0.26^{c}	71.63 ± 0.43^{b}	68.40 ± 0.32^{c}	<0.001
P Value	<0.001	<0.001	<0.001	
Extraction Temperature(˚C)				
4	11.48 ± 0.39^{d}	59.50 ± 0.45^{e}	55.49 ± 0.44^{e}	<0.001
15	13.54 ± 0.41^{c}	62.55 ± 0.34^{d}	57.46 ± 0.39^{d}	<0.001
25	14.46 ± 0.42^{c}	65.61 ± 0.55^{c}	59.36 ± 0.46^{c}	<0.001
35	16.70 ± 0.51^{b}	68.75 ± 0.44^{b}	63.44 ± 0.45^{b}	<0.001
50	19.47 ± 0.38^{a}	71.63 ± 0.52^{a}	68.34 ± 0.45^{a}	<0.001
P Value	<0.001	<0.001	<0.001	
W:V ratio				
1:20	12.44 ± 0.42^{e}	27.36 ± 0.55^{d}	26.69 ± 0.60^{d}	0.227
1:15	16.85 ± 0.46^{d}	28.70 ± 0.48^{d}	27.85 ± 0.50^{d}	0.101
1:10	18.52 ± 0.40^{c}	37.87 ± 0.82^{c}	34.54 ± 0.38^{c}	<0.003
1:6	24.80 ± 0.67^{b}	55.90 ± 0.87^{b}	48.79 ± 0.72^{b}	<0.001
1:3	27.71 ± 0.57^{a}	80.37 ± 0.42^{a}	64.63 ± 0.66^{a}	<0.001
P Value	<0.001	<0.001	<0.001	
Centrifugation speed (Rpm)				
2000	17.83 ± 0.69^{c}	70.96 ± 0.57^{b}	53.93 ± 0.72^{c}	<0.001
5000	20.22 ± 0.35^{b}	72.43 ± 0.54^{b}	61.27 ± 0.48^{b}	<0.001
8000	23.23 ± 0.59^{a}	75.99 ± 0.75^{a}	66.47 ± 0.57^{a}	<0.001
P Value	<0.001	<0.001	<0.001	
Stirring time				
Continuous stirring	20.44 ± 0.40^{a}	72.35 ± 0.51^{a}	59.99 ± 0.83^{a}	<0.001
Stirring every 15 min	19.81 ± 0.48^{a}	68.70 ± 0.51^{b}	57.88 ± 0.54^{b}	<0.001
Stirring every 30 min	17.42 ± 0.49^{b}	61.74 ± 0.51^{c}	51.12 ± 0.83^{c}	<0.001
Without stirring	14.40 ± 0.48^{c}	57.52 ± 0.43^{d}	48.61 ± 0.43^{d}	<0.001
P Value	<0.001	<0.001	<0.001	

Note: Different superscripts in small letters (a, b, c) indicate significant difference (p<0.05) amongst column. Value are expressed as mean ± SD (n=3)

Characterization of Pacu fish protein isolates obtained through pH shift process

Proximate composition

The proximate composition of Pacu isolates were significantly different (p<0.005) in both acidic and alkaline processing conditions (Table 4). Moisture, protein, fat, and ash content of the muscle meat used for the protein isolation was 75.62 ± 1.20, 18.36 ± 1.40, 1.32 ± 0.20 and 1.24 ± 0.15%, respectively in Pacu fish filleting waste. Alkaline processed isolate had higher protein content (22.87 ± 0.74%) than acidic processed isolates (21.01 ± 0.88) (p<0.005). Similar observation was made by [39, 45, 40], where the protein composition of rohu and pangas processing waste was 9.9 and 8.15 g/100 g, which fluctuated between 17.86 and 20.45 g/100 g in the isolates. There was an impulse towards higher moisture content in the isolates made with acid treating. This might stem from a higher water holding capacity of the acid administered proteins, something which can be attached to the unique unfolding-refolding pattern of the proteins when pointed to pH 2.5 vs. pH 10.8 before transition to pH 5.5 [32, 40] Another significant feature of protein isolate is the lipid composition which becomes diminished substantially in both acidic and alkaline processed isolate as reflected by 0.28 ± 0.10 (acidic) and 0.23 ± 0.74 (Alkaline) (p<0.005), while it was over 1% in filleting waste. Surasani, 2017, 2018, 2019 [39, 45, 40] reported that lipid content was reduced from 2.9 and 14.17 g/100 g in rohu and pangas processing waste to 0.24 and 0.78 g/100 g in isolates (p<0.05) which is a solid compliance with present results. The lipid levels in the isolates from the centrifugation process were in 3 out of 4 cases, actually small. It has been established earlier that membrane lipids can be ambushed in the bottom sediment

from centrifugation. However, since a small floating layer was also got after centrifugation, it is supposed that some neutral lipids partitioned into this layer. All these lipids can be removed well in centrifugation, by taking lead of density variations between lipids, water and proteins [50].

Color and whiteness

Colour values of Pacu fish protein isolate recovered by acidic/alkaline solubilisation process are showed in Table 4. Lightness (L*) and whiteness values were higher for acid processed isolates (54.80 ± 0.81 and 54.70 ± 0.80) than the values of alkaline processed isolates (54.80 ± 0.81 and 45.69 ± 2.06). It was noted that a* and b* value were higher in alkaline processed isolates compared to acid processed isolates (p<0.005). These results were supported by the findings of Surasani 2017, 2018, 2019 [39, 45, 40], who reported higher lightness value of acid processed isolates compared to alkaline processed isolates. Similar observations were also made by [51, 27, 32] during their investigations on rockfish, Atlantic croaker and Blue whiting, respectively. Surasani, 2018 [47] in the comprehensive review study reported whiteness values of isolates and gels ranging between 20 and 73, 20 and 74, and 35–76% for acid-aided process, alkaline-assisted process, and traditional surimi processing, respectively. Higher values of lightness in acid processed isolates could be due to greater removal of pigments, haemoglobin, myoglobin and melanin [34]. Acid process appears to cause dissociation of haemoglobin, forming colourless heme and globin thus by increasing the whiteness of acid processed isolates.

Table 4: Process characterisation of Pacu raw materials and the isolates obtained through pH shift processing

Characterisation	Pacu waste	Acid processed isolate (pH 2.0)	Alkali processed isolate (pH 12.0)	*P* value
Proximate composition (g/100 g)				
Moisture	75.62 ± 1.20^{Aa}	77.18 ± 1.80^{Aa}	76.17 ± 1.50^{Aa}	0.488
Protein	18.36 ± 1.40^{Bb}	21.01 ± 0.88^{Ab}	22.87 ± 0.74^{Ab}	0.005
Fat	1.32 ± 0.20^{Ac}	0.28 ± 0.10^{Bc}	0.23 ± 0.74^{Bc}	<0.001
Ash	1.24 ± 0.15^{Ac}	0.98 ± 0.14^{Ac}	0.80 ± 0.27^{Ac}	0.083
P value	<0.001	<0.001	<0.001	
Color values				
*L**	53.79 ± 1.69^{Aa}	54.80 ± 0.81^{Aa}	45.86 ± 2.08^{Ba}	<0.001
*a**	2.60 ± 0.36^{Ac}	0.95 ± 0.10^{Bc}	1.38 ± 0.35^{Bc}	<0.001
*b**	13.96 ± 0.69^{Bb}	8.37 ± 0.31^{Cb}	16.20 ± 0.53^{Ab}	<0.001
Whiteness	53.57 ± 1.66^{Aa}	54.70 ± 0.80^{Aa}	45.69 ± 2.06^{Ba}	<0.001
P value	<0.001	<0.001	<0.001	
Others attributes				
Total lipid content (%)	2.31 ± 0.48^{A}	0.35 ± 0.03^{B}	0.39 ± 0.14^{B}	<0.001
Total myoglobin content (mg/g)	278.41 ± 6.21^{A}	24.15 ± 1.30^{B}	22.58 ± 1.61^{B}	<0.001
Total pigment content (ppm)	176.46 ± 3.46^{A}	30.10 ± 1.85^{B}	32.60 ± 1.42^{B}	<0.001
Functional property				
Foaming capacity (ml/100 ml)	-	85.66 ± 2.73	64.82 ± 1.89	<0.001
Foaming stability (ml/100 ml)	-	55.36 ± 2.12	59.75 ± 2.84	0.098
Emulsion capacity (ml/100 ml)	-	56.51 ± 3.08	76.50 ± 2.95	<0.001
Water holding capacity (ml/g))	-	0.53 ± 0.18	1.71 ± 0.14	<0.001
Oil holding capacity (ml/g)	-	1.31 ± 0.34	2.57 ± 0.56	<0.028

Note: Different superscript in capital letters (A, B, C) indicate significant difference (p<0.05) amongst rows and superscripts in small letters (a, b, c) indicate significant difference amongst column. Value are expressed as mean ± SD (n=3).

Lipid, Pigment and Myoglobin content

The protein isolated obtained through acidic/alkaline aided processing had a reduced level of total lipid, myoglobin and pigments as compared to the raw material (Table 4). Higher lipid reduction was noted in acid processed isolates (0.35 ± 0.03%) compared to alkaline isolates (0.39 ± 0.14%) and Pacu mince (2.31 ± 0.48%). Similar trend was observed n myglobin and pigment content, which were maximum (278.41 ± 6.21 mg/g and 176.46 ± 3.46 ppm) in mince, followed by acidic isolates (24.15 ± 1.30 mg/g and 30.10 ± 1.85 ppm) and alkaline aided protein isolates (22.58 ± 1.61 mg/g and 32.60 ± 1.42 ppm), respectively (P<0.005). Surasni, 2018 [47] pointed out in his review that pH shift processing can remove efficiently more myoglobin from the proteins compared to conventional processing which is also supported by the findings of many researchers [7, 18, 20]. Rawdkuen, 2009 [18] reported lipid reductions of 85.2% for tilapia muscle concentrate and Kristinsson, 2005 [26] reported lipid reduction of 85.4% for catfish concentrate.

Myoglobin could be eliminated completely during alkaline–assisted processing of fish muscle [5]. Myoglobin contents were decreased efficiently in pH shift processing when related to conventional surimi processing [18]. Myoglobin extracting efficiency depends on species, muscle type, storage time and washing process [6]. High pigment removal in acid-aided processing might be due to the deterioration of haem pigments at extreme acid condition rather than discharge them from the isolate [34].

Functional properties

Functional properties such as *i.e.* foaming capacity (FC), foaming stability (FS), emulsion capacity (EC), water holding capacity (WHC), oil holding capacity (OHC) of acidic and alkaline processed Pacu isolates are depicted in Table 4. Foaming capacity was higher in acidic aided protein isolate than in alkaline based protein isolate, whereas foaming stability, water holding capacity and emulsion capacity were higher in alkaline processed isolate than acid processed isolates. Findings of the current investigation were supported by the reports of [39, 45], who interpreted the reason of these variations as the difference in hydrophobic residues of proteins. Similar observations were made by Freitas, 2011 [13] while extracting proteins from Argentine anchovy residue.

The low WHC of proteins explains the developments of the protein as the protein structure varies at extreme pH owing to

intermolecular repulsions, appearing in transformations in water holding capacity [16]. The high oil holding capacity of the isolates extracted using alkali extraction might be due to the lipid composition of the initial sample [13]. The variations in FC and EC are associated to the extent of hydrophobic residues in the proteins [43]. Acid and alkali extracted proteins from freshwater mussel had foaming capacities of 50.93 and 43.48%, respectively [45]. Pangas protein isolates extracted using acid and alkali process had foaming capacities of 66.29 and 54.50%, respectively [43]. The change in alkaline and acidic protein functionality might be due to the variations in hydrophobic amino acid content.

Properties of protein gels

Quality attributes of protein isolate gels are detailed in Table 5. Acid processed isolate gels had better color (whiteness) but poor texture compared to alkali processed isolate gels (p<0.005). Isolate gels followed the similar pattern of isolates in color characteristics. Gel strength values of alkaline and acid processed isolates were 265.41 ± 2.35 and 273.17 ± 2.88 g.cm., respectively. In contradiction to folding test, both isolate groups were found to be good with respect to folding test scores.

Table 5: Properties of protein gels prepared using Pacu protein isolates obtained through acid and alkaline solubilization

Gel properties		Acid processed isolate (pH 2.0)	Alkali processed isolate (pH 12.0)	*P* value
Colour	*L*	58.31 ± 1.08	50.28 ± 1.00	<0.001
	a	0.38 ± 0.18	0.84 ± 0.21	<0.042
	b	5.91 ± 0.46	13.33 ± 0.36	<0.001
	Whiteness	58.24 ± 1.07	50.14 ± 1.00	<0.001
Expressible moisture (g/100g)		11.77 ± 2.50	15.46 ± 2.18	0.126
Folding test		5.00 ± 0.00	5.00 ± 0.00	-
Gel strength (g.cm)		273.17 ± 2.88	265.41 ± 2.35	<0.022

Note: Value are expressed as mean ± SD (n=3).

Similar findings were reported by Surasani, 2017, 2018, 2019 [43, 45, 40], who reported that gels from acid processed isolates were weaker compared to the gels from alkali extracted isolates. The color values of isolate gels and isolates in this study was supported by the findings of Nolsøe, 2011 [32], who reported that the acid and alkaline processed blue whiting isolate gels had a whiteness value of 75.4 and 69.2, respectively. Similar observations were made by [33] during pH shift extraction of silver carp proteins. Chaijan, 2006 [5] explained that the acidic process caused poor networking of protein gels, because of excess protein denaturation at acidic pH. The influences that can determine the gel formation ability of proteins are fish species, mode of extraction and time of extraction [12]. Poor gelation characteristics of the acid aided protein isolates could be due to activation of some enzymes or additional proteolysis at acidic pH [27].

Amino acid profiling

Amino acid profile of muscle meat homogenate and protein isolates obtained through pH shift processing is provided in Table 6. In the present investigation, the effect of processing on amino acids was minimal and all the amino acids present in the homogenate were retrieved into isolates. Findings reveal that acidic/alkaline solubilisation process could not cause significant effect on the amino acid profiling (p>0.005) and all amino acid recovered effectively by pH process. Major amino acids in homogenate were GLY (Glycine), PHE (Phenylalanine), HIS (Histidine) and LYS (Lysine) with 9.12 ± 0.39, 8.52 ± 0.86, 30.22 ± 0.70 and 24.38 ± 0.60% respectively. The content of proline and glycine was less in acid-processed isolates followed by alkaline-processed isolates compared to homogenate, which might be due to non-extractability of connective tissues in alkaline processing [3]. The content of amino acid composition of Pacu processing waste little differs with species of Pacu in amino acid profiling [29].

Table 6: Amino acid profiling of raw material as well as proteins recovered from Pacu fillet frames during solubilization at pH 2.0 and 12.0.

Amino Acid (%)	Pacu waste	Acid processed isolate (pH 2.0)	Alkali processed isolate (pH 12.0)	*P* value
ASP	3.50 ± 0.82^{Ade}	4.12 ± 0.23^{Acd}	4.65 ± 1.09^{Ad}	0.283
THR[a]	4.05 ± 0.49^{Ad}	4.16 ± 0.76^{Acd}	4.20 ± 0.38^{Ade}	0.949
SER	1.87 ± 0.30^{Afg}	1.90 ± 0.83^{Ade}	2.12 ± 0.19^{Aef}	0.817
GLU	3.47 ± 0.65^{Ade}	4.12 ± 0.27^{Acd}	4.58 ± 0.82^{Ad}	0.171
PRO	0.22 ± 0.09^{Bh}	0.12 ± 0.03^{Be}	0.90 ± 0.25^{Af}	<0.002
GLY	9.12 ± 0.39^{Ac}	7.34 ± 0.51^{Bb}	3.87 ± 0.61^{Cde}	<0.001
ALA	0.40 ± 0.14^{Ah}	0.30 ± 0.13^{Ae}	0.22 ± 0.09^{Af}	0.254
CYS	0.24 ± 0.06^{Ah}	0.12 ± 0.05^{Be}	0.11 ± 0.04^{Bf}	0.037
VAL[a]	2.28 ± 0.41^{Cef}	3.12 ± 0.84^{ABcd}	4.14 ± 0.63^{Ade}	0.035
MET[a]	0.57 ± 0.12^{Bgh}	0.51 ± 0.07^{Be}	1.13 ± 0.21^{Af}	<0.004
I LEU[a]	2.21 ± 0.39^{Aef}	3.13 ± 0.23^{Acd}	3.87 ± 1.23^{Ade}	0.095
LEU[a]	3.11 ± 0.21^{Bdef}	4.86 ± 0.47^{Ac}	5.03 ± 0.37^{Ad}	<0.001
TYR	0.17 ± 0.04^{Bh}	0.30 ± 0.16^{Be}	0.65 ± 0.12^{Af}	<0.007
PHE[a]	8.52 ± 0.86^{Ac}	9.26 ± 0.44^{Ab}	9.50 ± 0.83^{Ac}	0.308
HIS	30.22 ± 0.70^{Aa}	25.48 ± 2.36^{Ba}	23.76 ± 1.20^{Bb}	<0.008
LYS[a]	24.38 ± 0.60^{Bb}	27.14 ± 1.06^{ABa}	27.88 ± 1.73^{Aa}	<0.028
ARG	0.36 ± 0.14^{Ah}	0.20 ± 0.10^{Ae}	0.33 ± 0.17^{Af}	0.389
TRP	----nd---	---nd---	---nd---	
P value	<0.001	<0.001	<0.001	

Note: Different superscript in capital letters (A, B, C) indicate significant difference (p<0.05) amongst rows and superscripts in small letters (a, b, c) indicate significant difference amongst column. Value are expressed as mean ± SD (n=3).nd= not determined. [a]Essential amino acid

ASP – Aspartic acid, THR – Threonine, SER – Serine, GLU – Glutamic acid, PRO – Proline, GLY – Glycine, ALA – Alanine, CYS – Cysteine, VAL – Valine, MET – Methionine, I LEU – Isoleucine, LEU – Leucine, TYR – Tyrosine, PHE – Phenylalanine, HIS – Histidine, LYS – Lysine, ARG – Arginine., TRP- Tryptophan

Similar findings were observed by [39, 44] during their study on protein isolates recovered from pangas waste. They noticed that the pH shift processing recovered all the amino acids present in the homogenate and the pH shift process caused enhancement of essential amino acids in the protein isolates. However, the final amino acid content of the protein isolates obtained through pH shift processing depends on raw material used and the pH at which the protein was solubilized. Quality of protein is determined by the presence and abundance of essential amino acids. Isolates obtained using alkaline processing had a high amount of amino acids than isolates collected by acid treatment. High amino acid composition of alkaline processed isolates might be due to less pH-induced proteolysis in alkaline processing [14]. The overall amino acid composition in Pacu protein isolates obtained by pH shift processing was well above the suggested standards for adults by FAO/WHO/UNO. Marmon and Undeland, 2010 [32] published identical conclusions during their subjects on protein isolates recovered from herring through pH shift processing.

Conclusion

Protein isolate recovered from Pacu fish by acid/alkali solubilization method have showed good yield and functional properties of protein. Maximum solubility of Pacu proteins was found at pH 2.0 and pH 12.0 which produced isolates with good functionality. The alkaline aided protein isolation was proved to be better in terms of protein yield as well as quality of isolates. The recovered proteins showed higher protein concentrate, gelling and texture properties as well as good amino acid profile. Although technology is well accepted for the recovery of proteins from marine fish processing waste, there is a need to find some alternate technology to reduce the amount of alkali and acid used in this process. Moreover, application of recovered proteins in edible systems is still at an initial stage, which needs extra efforts and thorough research in this area.

Acknowledgements

Authors wish to express their sincere thanks to the Dean, College of Fisheries, KVAFSU, Mangalore, for their constant support during the work period. Authors also acknowledge to UGC for granting funding under the scheme of National Fellowship for Other Backward Classes (NFOBC) to the PhD research.

References

1. Singh AK. Apprehensions and issues related to Pacu *Piaractus brachypomus* (Cuvier 1818) farming in India. J Fish Res. 2018; 2(1):19-23.
2. AOAC. Official Methods of Analysis of AOAC International. Association of Official and Analytical Chemists, International Virginia, USA, 2000.
3. Batista I. Recovery of proteins from fish waste products by alkaline extraction. European Food Research and Technology. 1999; 210:84-89.
4. BIdlingmeyer BA, Cohen SA, Tarvin TL. Rapid analysis of amino acids using precolumn derivatisation. Journal of Chromatography. 1984; 336:93-104.
5. Chaijan M, Benjakul S, Visessanguan W, Faustman C. Physicochemical properties, gel-forming ability and myoglobin content of sardine (*Sardinella gibbosa*) and mackerel (*Rastrelliger kanagurta*) surimi produced by conventional method and alkaline solubilisation process. Journal of European Food Research and Technology. 2006; 222:58-63.
6. Chaijan M, Panpipat W, Benjakul S. Physicochemical and gelling properties of short-bodied mackerel (*Rastrelliger brachysoma*) protein isolate prepared using alkaline-aided process. Food and Bio products Processing. 2010; 88:174-180.
7. Chen HH. Effect of cold storage on the stability of chub and horse mackerel myoglobins. Journal of Food Science. 2003; 68:1416-1419.
8. Choi YJ, Park JW. Acid aided protein recovery from enzyme-rich Pacific whiting. Journal of Food Science. 2002; 67:2962-2967.
9. FAO. The state of world fisheries and aquaculture, Food and Agriculture Organization, Rome, 2020.
10. Feng YM, Hultin HO. Effect of pH on the rheological and structural properties of gels of water-washed chicken-breast muscle at physiological ionic strength. Journal of Agricultural and Food Chemistry. 2001; 49:3927-3935.
11. Foh MBK, Wenshiu X, Amadou I. Jiang Q. Influence of pH Shift on Functional Properties of Protein Isolated of Tilapia (*Oreochromis niloticus*) Muscles and of Soy Protein Isolate. Food and Bioprocess Technology. 2011; 5(6):1-9.
12. Freitas IR, Cortez-Wega WR, Prentice C. Evaluation of properties of protein recovered from fish muscle by acid solubilization process. Int Food Res J. 2015; 22(3):1067-1073.
13. Freitas IR, Gauterio GV, Rios DG, Prentice C. Functionality of protein isolates from Argentine anchovy (*Engrauli sanchoita*) residue using pH shift processing. Journal of Food Science and Engineering. 2011; 1:374-378.
14. Gehring CK, Gigliotti C, Moritz JS, Tou JC, Jaczynsky J. Functional and nutritional characteristics of protein and lipid recovered by isoelectric processing of fish by-products and low-value fish: A review. Food Chemistry. 2011; 124(2):422-431.
15. Hamm R. The influence of pH on the protein net charge in the myofibrillar system. Reciprocal Meat Conference Proceedings. 1994; 47:5-9.
16. Hultin HO, Kelleher SD. Process for Isolating a Protein Composition from a Muscle Soure and Protein Composition. U.S. Patent 6,005,073, 1999.
17. Hultin HO, Kristinsson HG, Lanier Tyre C, Park JW. Process for Recovery of Functional Proteins by PH-shifts. In: ParkSurimi and surimi seafood, Boca Raton; Taylor and Francis Group, 2005, 107-139.
18. Rawdkuen S, Sai-ut S, Khamsorn S, Chaijan M, Benjakul S. Biochemical and gelling properties of tilapia surimi and protein recovered using an acid-alkaline process. Food Chemistry. 2009; 112**:**112-119.
19. Ibrahim, HMI. Chemical composition, minerals content, amino acids bioavailability and sensory properties of meat and fish balls containing fish protein isolate. International Journal of Current Microbiology and Applied Sciences. 2015; 4:917-933.

20. Jafarpour SA, Shabanpour B, Filabadi SS. Biochemical properties of fish protein isolate (FPI) from silver carp (*Hypophthalmichthys molitrix*) by application of acid-alkali processes compared to traditional prepared surimi. Ecopersia. 2013; 1(3):315-327.
21. L, Berk Z, Pariser ER, Goldblith SA, Flink IK. Squid protein isolate: Effect of processing conditions on recovery yields. Journal of Food Science. 1974; 39:592-595.
22. Kain RJ, Chen Z, Sonda TS, Abu-Kpawoh JC. Study on the effect of control variables on the extraction of peanut protein isolates from peanutmeal (*Arachis hypogaea* L.). American Journal of Food Technology. 2009; 4(1):47-55.
23. Kelleher SD, Hultin HO. Functional chicken muscle protein isolates prepared using low ionic strength, acid solubilization/precipitation. 53rd Annual Reciprocal Meat Conference proceedings, 1994, 76-81.
24. Kristinsson HG, Theodore AE, Ingadottir B. Chemical Processing Methods for Protein Recovery from Marine By-products and Underutilised Fish Species, in Shahidi, Maximizing the Value of Marine By-products, Cambridge, Woodhead, 2006, 144-168.
25. Kristinsson H and Ingadottir B. Recovery and properties of muscle proteins extracted from tilapia (*Oreochro misniloticus*) light muscle by pH shift processing. Journal of Food Science. 2006; 1(3): E132-E141.
26. Kristinsson H, Theodore AE, Demir N, Ingadottir B. A comparative study between acid- and alkali-aided processing and surimi processing for the recovery of proteins from Channel catfish muscle. Journal of Food Science. 2005; 70(4):C298-C306.
27. Kristinsson HG, Liang Y. Effect of pH-shift processing and surimi processing on Atlantic croaker (*Micropogonias undulates*). Journal of Food Science. 2006; 71(5):304-312.
28. Kudo G, Okada M, Miyauchi D. Gel-forming capacity of washed and unwashed flesh of some Pacific coast species of fish. Marine Fisheries Reviews. 1973; 32:10-15.
29. Murthy LN, Madhusudana BR, AshaKK, Prasad MM. Nutritional composition, product development, shelf-life evaluation and quality assessment of Pacu *Piaractus brachypomus* (Cuvier, 1818). Indian J. Fish. 2015; 62(1):101-109, 2015.
30. Marmon SK, Undeland I. Protein isolation from gutted herring (*Clupea harengus*) using pH-shift processes. Journal of Agricultural and Food Chemistry. 2010; 58:10480-6.
31. J, Constantinides SM, Yang CST. Optimization of processing parameters for the preparation of flounder frame protein product. Journal of Food Science. 1984; 49:172-176.
32. Nolsøe H. Protein Yield and Protein Isolate Quality when applying pH-shift Processing on Cod, Haddock and Blue Whiting Fillets; Effects of replacing centrifugation with filtration. Technical Report no 2011:6. Dept. Chemical and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden, 2011.
33. Paker I, Beamer S, Jaczynski J, Matak KE. pH shift protein recovery with organic acids on texture and color of cooked gels. Journal of the Science of Food and Agriculture. 2015; 95(2):275-80.
34. Panpipat W, Chaijan M. Biochemical and physicochemical characteristics of protein isolates from bigeye snapper (*Priacanthus tayenus*) head by-product using pH shift method. Turkish Journal of Fisheries and Aquatic Sciences. 2016; 6:41-50.
35. S, Saiut S, Khamsorn S, Chaijan M, Benjakul S. Biochemical and gelling properties of tilapia surimi and protein recovered using an acid-alkaline process. Food Chemistry. 2009; 112:112-119.
36. Reddy SVK. Effect of formulation and processing methods on the quality and acceptability of cutlets made from minced meat of Pangas (*Pangasius pangasius*). SAARC Journal of Agriculture. 2016; 14(1):25-36.
37. Sathe SK, Deshpande SS, Salunkhe DK. Functional properties of lupin seed (*Lupinus mutabilis*) proteins and protein concentrates. Journal of Food Science.1982; 7:191-19.
38. Shen JL. Soy protein solubility: The effect of experimental conditions on the solubility of soy protein isolate. Cereal chemistry. 1975; 53(6):902-909.
39. Surasani VKR. Influence of Rohu (*Labeo rohita*) deboning by-product on composition, physical properties and sensorial acceptability of rohu cutlets. Nutrition & Food Science. 2017; 47(3):398-408.
40. Surasani VKR, Arashdeep S, Antima G, Savita S. Functionality and cooking characteristics of pasta supplemented with protein isolate from Pangas processing waste. LWT. 2019; 111:443-448.
41. Surasani VKR, Raju CV, Chandra M, Uzairshafiq, Lakshmisha IP. Effect of organic acid on recovery yields and characteristics of rohu (*Labeo rohita*) protein isolates extracted using pH shift processing. Journal of the Science of Food and Agriculture. 2020; 99(14):6546-6551.
42. Surasani VKR, Raju CV, Uzairshafiq, Chandra MV, Lakshmisha IP. Influence of protein isolates from Pangas processing waste on physico-chemical, textural, rheological and sensory quality characteristics of fish sausages. LWT - Food Science and Technology. 2020; 111:443-448.
43. Surasani VKR, Khatkar SK, Singh S. Effect of process variables on solubility and recovery yields of proteins from pangas (*Pangasius pangasius*) frames obtained by alkaline solubilization method: Characteristics of isolates. Food and Bioproducts Processing. 2017; 106:137-146.
44. Surasani VKR, Kudre T, Ballari RV. Recovery and characterization of proteins from pangs (*Pangasius pangasius*) processing waste obtained through pH shift. Environmental Science and Pollution Research. 2018. https://doi.org/10.1007/s11356-018-1456-x.
45. Surasani VKR, Mandal A, Pandey A. Utilization of freshwater mussel (*Lamellidens marginalis*) for the isolation of proteins through pH shift processing: characterization of isolates. Environmental Science and Pollution Research. 2018; 25(31):31497-31507.
46. Surasani VKR, Tyagi A, Kudre T. Recovery of Proteins from Rohu Processing Waste Using pH Shift Method: Characterization of Isolates. Journal of aquatic food product technology. 2016; 26(3):356-365.
47. Surasani VKR, Acid and alkaline solubilization (pH shift) process: a better approach for the utilization of fish processing waste and by-products. Environmental Science and Pollution Research, 2018. https://doi.org/10.1007/s11356-018-2319-1
48. Thorkelsson G, Sigurgisladottir S, Geirsdottir M, Jóhannsson R, Guérard F, Chabeaud A *et al*. Mild Processing Techniques and Development of Functional

Marine Protein and Peptide Ingredients. In Børresen, Improving seafood products for the consumer, Wood head Publishing Limited, 2008.
49. Undeland I, Kelleher SD, Hultin HO. Recovery of Functional Proteins from Herring (*Clupea harengus*) Light Muscle by an Acid or Alkaline Solubilization Process. Journal of Agricultural and Food Chemistry. 2002; 50(25):7371-7379.
50. Vareltzis PK, Evaggelia P, Ntoumas D, Adamopoulos KG. Process characteristics and functionality of sardine (*Sardina pilchardus*) muscle proteins extracted by a pH-shift method. Annals in Food Science and Technology. 2012; 13(2):132-143.
51. Yongsawatdigul J, Park JW. Effects of alkali and acid solubilization on gelation characteristics of rockfish muscle proteins. Journal of Food Science. 2004; 69(7):499-505.
52. Robinson HW, Hogden CG. The biuret reaction in the determination of serum proteins. J Biol. Chem. 1940; 135:707-725.