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Comparing Efficiency of Green Methods for Surimi Skin and Bone Gelatin Extraction

KHUSHBOO[1,2], NUTAN KAUSHIK[1]*, KRISTINA NORNE WIDELL[3], RASA SLIZYTE[3] and ASHA KUMARI[1]

[1]Amity Food and Agriculture Foundation, Amity University Uttar Pradesh, Noida 201313, India.
[2]Amity Institute of Food Technology, Amity University Uttar Pradesh, Noida 201313, India.
[3]Department of Fisheries and New Biomarine Industry, 7465, SINTEF Ocean, Trondheim, Norway.

Abstract

Globally, the surimi processing industry produces a large amount of by-products in the form of head and viscera, skin, bones, scale, etc. The aim of this study was to assess the combined biomass of pink perch skin and bones obtained from the surimi industry as a potential source of raw material for gelatin production and identify a green method of gelatin extraction by comparing four green processes. Four green gelatin extraction processes were compared for their gelatin extraction efficiency. Among the four processes, process 1 and process 2 comprised of two-step extraction viz pre-treatment with NaCl and extraction with hot water. Process 3 and process 4 comprised of single-step wherein pre-treatment and extraction were done simultaneously with acidic water using acetic acid. The gelatin extraction efficiency was determined based on the yield and L-hydroxyproline content of the extracted gelatin. Further, the extracted gelatin was characterized for their proximate and amino acid composition. The acetic acid based single-step method was found to be more efficient in the extraction of gelatin than the NaCl pretreatment method. The gelatin extracted with this method had a higher yield (4.2%), protein content (79.6%), and imino acid (27.3%) content than the NaCl pretreatment method, which had 1.51% gelatin yield, 48.1% protein content, and 13.1% imino acid content. The results suggested that the single-step extraction method can be effectively utilized for the extraction of gelatin from pink perch skin and bones combined biomass. This study provides a method for the valorization of the surimi industry by-product into a high value product with potential application in various industries.

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CONTACT Nutan Kaushik kaushikn2008@gmail.com Amity Food and Agriculture Foundation, Amity University Uttar Pradesh, Noida 201313, India.

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Introduction

Surimi is a fish mince product widely consumed throughout the world to manufacture imitation meat such as crab sticks and fish balls. In 2021, global surimi market size was valued at USD 5535.5 million and growing at rate of 8.2% CAGR, is expected to reach USD 9672.1 million by 2028.[1] Surimi is obtained after repeated washing of the flesh part to eliminate lipids, sarcoplasmic protein, blood, and enzymes to extract myofibrillar proteins.[2] Globally, more than 60% of surimi is produced using tropical fishes such as pink perch.[3] Properties such as lean meat, white flesh, high gelling capacity, low cost, and abundance make it suitable for surimi production. During surimi processing, around 60% of the live weight of fish which included head and viscera, skin and bones, roe, scales fins, etc., is discarded as low-value by-products.[4] Around 30% (~ 1,100,000 T) of this by-product is produced as a mixture of skins, and bones embedded together. Since this biomass leads to operational difficulty in separation and further processing, it is currently either being utilized to produce low-value commercial products such as fishmeal, animal feed, and fertilizers or landfilled and discharged back to the water.[3] Discarding of the huge amount of this valuable biomass has resulted in increased organic load and contributed to environmental issues. Extracting biologically relevant molecules such as gelatin or collagen from the by-products provides a sustainable alternative approach for reducing waste and increasing economic benefits.

Gelatin is a functional biopolymer produced by the partial denaturation of collagen molecules. It is a versatile material with various applications in the food, pharmaceutical, and cosmetics industries.[3] Conventionally, mammalian gelatin is utilized but due to the increasing demand for gelatin in the global market, alternative sources of gelatin are being explored. Marine sources for gelatins provide a culturally acceptable and environmentally sustainable alternative source of gelatin.[5,6]

The typical gelatin extraction method includes more than two steps i.e., a pretreatment step followed by extraction, which makes the process time-consuming, cost-intensive, and cumbersome.[7,8] Multi-step processes increase the number of parameters affecting the yield and qualitative characteristics of gelatin, which are already affected by the source of raw material and the type of tissue used for extraction. Conventionally, pink perch (Nemipterus japonica) gelatin involves extraction from either skin or bones separately using multi-step approaches and harsh chemicals which may be detrimental to the environment.[9,10,11] Therefore, it may be desirable to adopt environmentally sensitive techniques to extract gelatin sustainably and efficiently. Based on the fifth principle of green chemistry i.e., the use of safer chemicals and reaction conditions, this study aims at providing a green method of gelatin extraction by utilizing green solvents viz. acetic acid and sodium chloride,[12,13] and therefore, eliminating the use of hazardous mineral acids. Mineral acids are corrosive in nature and cause negative impacts on the environment and occupational safety. Further, the reduction of the number of steps of the extraction process provides a more controlled reaction and end product. This also facilitates quicker gelatin extraction with minimum losses.

This research aimed to evaluate the suitability of combined biomass of pink perch skin and bones from the surimi industry for gelatin extraction and compare the extraction efficiency of single step gelatin extraction process with multi-step gelatin extraction process.

Material and Methods

Material

The frozen combined biomass of pink perch skin and bones, obtained from belt and drum type deboner, was procured from Refrigerated Distributors Pvt. Ltd., Mumbai, India in frozen gel packing. The sample was thawed at room temperature for 1 h and homogenized using a Hobart mincer (AE 200, Hobart, Ohio, USA) fitted with a sieve of 10 mm hole size. The minced biomass was stored at -20°C till further experimentation. Glacial acetic acid, sodium chloride, and sodium hydroxide were procured from Thermo Fischer Scientific Ltd., Mumbai, India. Analytical grade chemicals and reagents were used for this study.

Proximate Analysis

The chemical composition (moisture, fat, protein, ash) of skin and bones biomass was determined using AOAC methods 950.46, 963.15, 928.08, and 938.08, respectively.[14]

Moisture Content Estimation

The moisture content of the skin and bone biomass was estimated using the hot air oven method. An empty dish was dried in the oven at 105°C for 3 h and then transferred to a desiccator for cooling. The weight of the empty dish was recorded and 10 grams of sample were placed on it. The sample and the dish were kept in a hot air oven for 24 hours at 105°C. The sample and the dish were kept in a desiccator for cooling prior to reweighing. The moisture content of the sample was calculated as the following equation (1).

$$\text{Moisture Content (\%)} = (W2-W1)/W1\times100 \quad ...(1)$$

Where

W1 – initial weight of fresh the sample (g)
W2 – weight of the dried sample (g)

Fat Content Estimation

Fat content estimation was carried out using the Soxhlet method. For measurement of fat content, a moisture-free sample (2 g) was taken into an extraction thimble and extracted with n-hexane in a soxhlet apparatus for over 8 hours. The remaining solvent was removed by evaporation. After drying at 80°C, the flask was cooled and the final weight was recorded. The fat content of the sample was calculated as the following equation (2).

$$\text{Fat Content (\%)} = (W1-W2)/W3\times100 \quad ...(2)$$

Where,

W1 – weight of extraction flask with fat (g)
W2 - weight of extraction flask (g)
W3 - weight of sample (g)

Crude Protein Estimation

The crude protein content of the skin and bone biomass was determined by total nitrogen content (N), estimated using the Kjeldahl method. 0.3 g of sample was digested with 4 g of digestion mixture (Potassium sulphate: copper sulphate, (5:1)) with 10 mL of concentrated sulphuric acid. The digestion was continued at 420°C till a clear green color solution was obtained. After cooling, the solution was diluted using distilled water and then distilled with 4% boric acid and 40% sodium hydroxide. Released ammonia was absorbed in boric acid solution containing mixed indicator (methyl red and bromocresol green, (1:1)). The distillate was then titrated against 0.1 N Hydrochloric acid in presence of methyl red indicator. The nitrogen-to-protein conversion factors used for the skin and bones biomass sample,[15] and gelatin sample[16] were 6.25 and 5.55, respectively. The protein content of the sample was calculated as the following equation (3 & 4).

$$\text{\% Nitrogen(N)} = (1.4 \times 0.1\ N \times V)/(W) \quad ...(3)$$

V - Titration value (ml),
W- Weight of sample (g)

$$\text{Protein Content (\%)} = \%N \times 6.25/5.55 \quad ...(4)$$

Ash Content Estimation

Ash content was analyzed by the gravimetric method. Ashing of the sample was done by weighing 2 g sample in a crucible and burning the sample at 600°C for 24 h in a muffle furnace. After ashing the crucible were cooled in a desiccator and weighed. The ash content of the sample was calculated as the following equation (5).

$$\text{Ash content (\%)} = (W1-W2)/W3\times100 \quad ...(5)$$

Where

W1 – Weight of crucible with residue
W2 - Weight of empty crucible
W3 – Weight of the sample

Scanning Electron Microscopy-Energy Dispersive X-Ray Analysis (SEM-EDX)

For SEM-EDX analysis ash of the sample was coated on a double-sided carbon tape (Thermo Fischer Scientific Ltd., Mumbai, India). The extra sample was dusted off to ensure a fine layer of the sample (2-3 mm) on the carbon tape. The sample was then further coated with gold-palladium mixture under a vacuum for 4 min using a sputter coater (SC7620, Quorum Technologies, Ltd., East Sussex, UK). The microstructure of the sample was observed in a Scanning Electron Microscope (SEM) (MA EVO -18 Special Edition, Zeiss India, Bangalore, India) at 20 kV acceleration voltage with magnification (1000X and 10,000X). Elemental confirmation was done on the cross-section of the scale using an EDX analyzer (EDS, Oxford Instruments, UK) connected to SEM.

Microbiological Study

Aerobic plate count, yeast and mold count, *Salmonella* count, and *E. coli* count were enumerated for assessment of microbial load in the sample. The homogenized sample (1 g) was serially diluted from 10^{-1} to 10^{-6} using 1% peptone water. 100 µL of inoculum was plated on different agar plates using the spread plate technique. Media used for determination of aerobic plate count, yeast and mold count, *E. coli* count, and *Salmonella* count was nutrient agar, Czapek Dox agar, Eosin Methylene Blue agar, and Xylose Lysine Deoxycholate agar, respectively. The inoculated plates of the aerobic plate, *Salmonella*, and *E. coli* count were incubated at 37°C for 24 h, and yeast and mold count plates were incubated at 25°C for 72 h. The results were expressed in CFU.g^{-1}

Gelatin Extraction

Four green processes (process 1 to process 4) were designed based on the literature for the extraction of the gelatin to compare their extraction efficiency and ease of preparation. Process 1 and process 2 were two-step extraction processes involving pretreatment followed by water extraction whereas in process 3 and process 4, pre-treatment and extraction methods were combined as a single step. Detailed methodology is provided below.

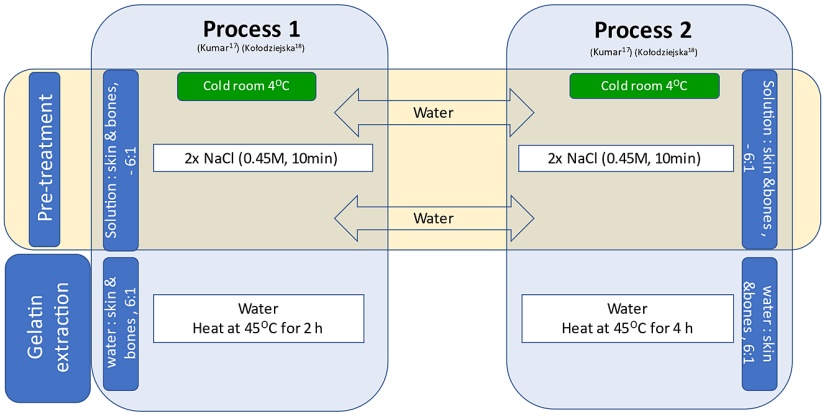


Fig. 1: Schematic representation of gelatin extraction in two steps extraction with NaCl pre-treatment.

Two Steps Extraction wth Nacl Pre-Treatment – Process 1 and Process 2

Extraction of the gelatin with NaCl pre-treatment was performed as per the methods of Kumar[17] and Kołodziejska[18] with slight modifications. The minced biomass (25 g) was thoroughly rinsed with distilled water and combined with 0.45 M NaCl solution in a 1:6, w/v ratio for 10 min at 4°C in an ice bath (Figure 1). The contents were filtered and washed with cold (10°C) distilled water. The process was repeated twice.

For the extraction of gelatin, the pre-treated biomass was mixed with the water in a ratio of 1:6, w/v. For process 1, the mixture was heated at 45°C for 2 h, and process 2, the mixture was heated at 45°C for 4 h with constant mixing (Figure 1). After heating the gelatin extract was filtered, and the filtered liquid gelatin was freeze-dried using a lyophilizer (SNS FD-50, S N Solutions, Noida, India) at -40°C. The lyophilized gelatin was stored at -20°C until further use.

Single-Step Extraction with Acetic Acid and Water- Process 3 and Process 4

Gelatin extraction using acetic acid was done according to the method of Derkach[19] with slight modifications. Pink perch skin and bones biomass mince (25 g) was thawed and mixed in a 1:3 w/v ratio with distilled water. Extraction was carried out by adjusting the pH of the solution to pH 3 (process 3) and pH 5 (process 4) using glacial acetic acid and incubating the contents at 50°C for 3 h (Figure 2). The pH of the solution was neutralized to pH 6.0-6.5 using 4M NaOH and filtered. The filtrate was freeze-dried using a lyophilizer (SNS FD-50) at -40°C. The lyophilized gelatin was stored at -20°C until further use.

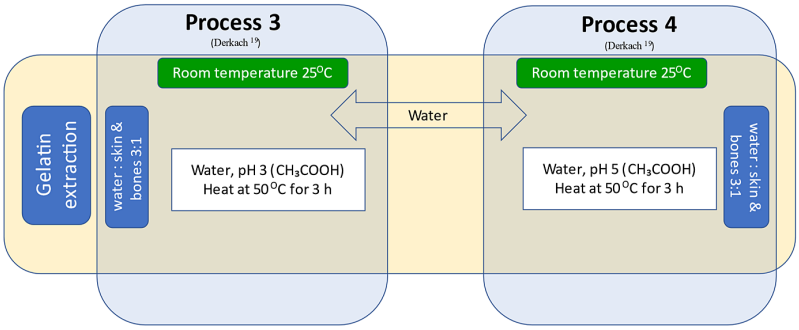


Fig. 2: Schematic representation of gelatin extraction in single-step extraction with acetic acid and water.

L- Hydroxyproline Content Estimation

L- Hydroxyproline (L- Hyp) content of the extracted gelatin was estimated as per the method of Bergman and Loxley.[20] The gelatin sample was hydrolyzed for 3 h at 110°C using 12M HCl and the contents was filtered. The filtrate was neutralized using 1M NaOH. The neutralized sample was oxidized with isopropanol oxidant solution (aqueous solution of 7%, w/v, chloroamine T mixed with pH 6 acetate/citrate buffer, in a ratio of 1:4, v/v) and incubated with Ehlrich reagent (2 g of p-dimethylamino benzaldehyde mixed in 3 mL of 60%, v/v perchloric acid) and 13 mL of isopropanol) at 60°C for 3 h. Absorbance was recorded using a UV-VIS spectrophotometer (LMSPU1000B, Labman Scientific Instruments Pvt. Ltd., Chennai, India) at 558 nm. The calibration curve was prepared with L-hydroxyproline (99.0%) (Sisco Research Laboratories Pvt. Ltd., Taloja, India). The concentration of the standard solution ranged from 0.01 $mg.g^{-1}$ to 0.1 $mg.g^{-1}$. The calibration curve was used for calculating L-hydroxyproline content in mg/g.

Yield Percentage

Protein yield (PY), gelatin yield (GY), and product yield of the gelatin was calculated as per the method of Tümerkan.[21] PY was determined by the ratio of the protein content in gelatin extract and the protein content in fresh skin and bones biomass. GY was defined as the amount of L-hydroxyproline in gelatin extract in comparison to the amount of L-hydroxyproline in fresh skin and bones biomass. Product yield (%) was calculated as follows-

Product yield (%)=100× (Weight of lyophilised gelatin (g))/(Weight of initial fresh sample (g))

Amino Acid Composition

For amino acid analysis, the gelatin sample (100 mg) was hydrolyzed using 2 mL of 6M HCl at 110ºC for 24 h.[4] The mixture was further neutralized with 6M NaOH and filtered. The volume of the filtrate was made-up to 5 mL using HPLC grade water (Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India), and the solution was filtered using a 0.22 µm PVDF membrane syringe filter (Durapore, Merck Life Sciences Pvt. Ltd., Vikhroli, India). Online-pre-column derivatization of the amino acid was done with OPA as a derivatizing reagent. The HPLC system (Agilent Infinity 1260, Agilent Technologies, CA, USA) coupled to a fluorescent detector (FLD) and a Zorbax eclipse AAA column (4.6×150 mm, 3.5 µmm) (Agilent Infinity 1260, Agilent Technologies, CA, USA) was used for amino acid analysis. The mobile phase consisted of a gradient of solution A (Disodium hydrogen phosphate + sodium tetraborate) and solution B (Methanol/Acetonitrile/ Water (45:45:10)). Standard curves were developed with amino acid standards procured from Agilent Technologies, CA, USA to quantify the amino acids.

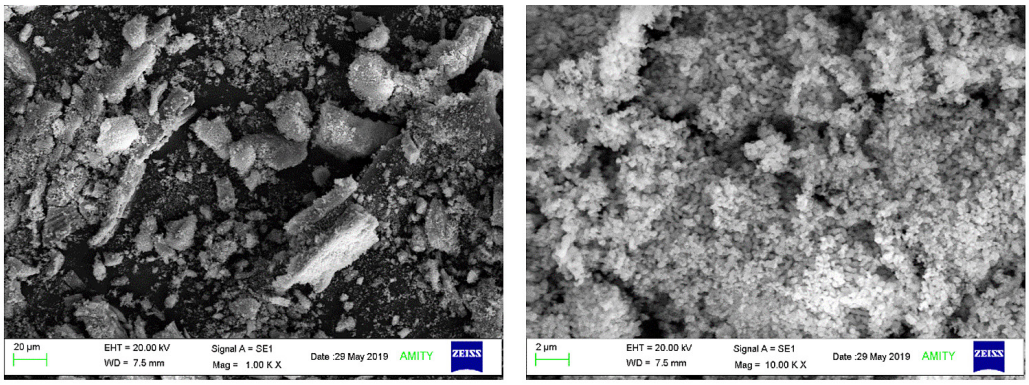
Statistical Analysis

All the experiments were done in triplicates and the results are presented as mean ± S.D. The data were analyzed statistically using one-way ANOVA. The significance of the mean difference was determined by Duncan Multiple Range Test (DMRT) using IBM Statistical Package for Social Sciences (SPSS) (Version 26.0, IBM India Pvt. Ltd., Bengaluru, India). The level of significance of the current study was $p<0.05$.

Results and Discussion

Proximate Analysis of Skin and Bones Biomass

Proximate composition determined the major components of the skin and bones biomass, which aids in determining further processing required for their utilization. The combined biomass of pink perch skin and bones recorded 73.1% moisture; 1% fat; 13.3% protein and 10.2% ash content. Results obtained are in close agreement with previously reported results of other by-products (head, skin, and internal organs) from the threadfin beam family i.e., 13.8-19.7% protein, 0.6-4.2% lipid, 2.2-11.9% ash and 69.4-77.5% moisture.[22] Since pink perch is a lean fish therefore low-fat content was observed in skin and bones biomass. The result indicated the presence of a high amount of protein in the combined biomass of skin and bones, suggesting that it can also be used to extract value-added products, such as gelatin.



(a) **(b)**

Fig. 3: Microstructure of pink perch skin and bone combined biomass (a) at 1000 magnification, (b) at 10,000 magnification

Table 1: Mineral elements present in pink perch skin and bones combined biomass

Elements	Weight (%)
Oxygen	41.0±4.0
Calcium	32.0± 5.0
Phosphorus	18.0±1.0
Carbon	6.2±0.6
Sodium	1.2±0.1
Magnesium	1.0±0.1
Potassium	1.0±0.1
Chlorine	0.2±0.2
Aluminum	0.00
Silicon	0.00
Tungsten	0.00

*Values are represented as means ± the SD.

Scanning Electron Microscopy-Energy Dispersive X-Ray Analysis (SEM-EDX)

The microstructure of fish skin and bones was visualized using SEM (Figure 3). Skin and bones are composed of irregularly shaped aggregates of

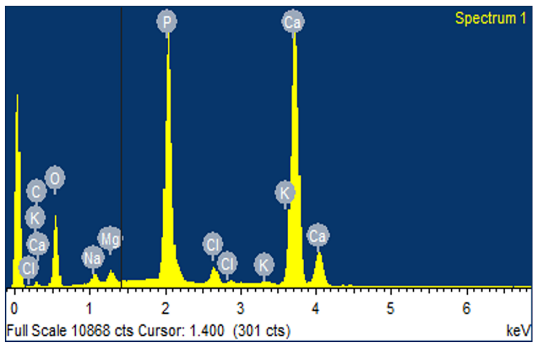


Fig. 4: Electron Dispersive X-Ray of pink perch skin and bones combined biomass

crystals. It showed the presence of elements such as Ca, P, C, O, and Na (Table 1, Figure 4). Calcium and phosphorus were major inorganic components, whereas, oxygen and carbon were the dominant organic matter of skin and bone by-products.

EDX is a surface analytical method for identification of the elemental composition of a sample. EDX was conducted for the combined biomass of skin and bones, confirming the presence of alkali and alkali

earth metals and the absence of any heavy metal. Therefore, the skin and bones of pink perch obtained from the surimi industry can be further utilized for processing into value-added products.

Microbiological Assessment of Skin and Bones Biomass

The results for the microbial load of pink perch skin and bones are provided in Table 2. The maximum permitted microbiological limit as per the Food Safety and Standards Authority of India (FSSAI) for frozen finfish for aerobic plate count is 1.0×10^7 CFU.g^{-1}, *E. coli* is 500.0 CFU.g^{-1}, absence of *Salmonella* sp. The aerobic plate count, *Salmonella* count, *E. coli* count and yeast and mold count for pink perch skin and bones were 1.4×10^7 CFU.g^{-1}, 0.0 CFU.g^{-1}, 0.0 CFU.g^{-1}, and 3.1×10^6 CFU.g^{-1}, respectively. The microbial count of the investigated sample was slightly above the maximum acceptable limit for aerobic plate count and within the permissible limits for *E. coli* and *Salmonella* indicating the absence of pathogenic micro-organisms. Acceptable microbial quality suggests the collection of samples from a pollution-free water source and also the maintenance of proper aseptic handling and processing conditions throughout the processing. Thus, the by-products received from industry can be utilized for further processing to develop value addition for human consumption.

Table 2: Aerobic plate count, *Salmonella count, E. coli* count, and Yeast and mold count of pink perch skin and bones combined biomass

Parameter	Pink perch skin and bones CFU.g^{-1}	Permissible Limit* CFU.g^{-1}
Aerobic plate count	1.4×10^7	1.0×10^7
Salmonella count	0.0	0.0
E. coli count	0.0	500.0
Yeast and mold count	3.1×10^6	NA

*Permissible Limit of the Food Safety and Standards Authority of India (FSSAI, 2011)
Values are represented as means ± the SD. NA: Not Available

Comparison of gelatin different extraction methods

Various methods reported so far for the gelatin extraction using fish skin and bones sample involve a multi-step to minimum two-step extraction process i.e., pre-treatment with acid /alkali followed by extraction with hot water.[6,21,23] This makes them cumbersome and time-consuming processes. In the present study, we compared four different processes of gelatin extraction (process 1, process 2, process 3, and process 4) from pink perch skin and bones combined biomass. Process 1 and process 2 comprised of two-step extraction viz pre-treatment with NaCl and extraction with hot water however, they differed only in extraction time. Process 3 and process 4 comprised of single-step wherein pre-treatment and extraction were done simultaneously with acidic water using acetic acid however the pH of the extraction system was different. Therefore, in comparison to previous studies the current study provided additional benefits by utilizing green solvents such as sodium chloride and acetic acid, therefore, eliminating the use of sodium hydroxide, sulphuric acid etc. Additionally, reduced number of processing steps improved control over the process and decreased overall losses during extraction (Table 3). In previously reported studies, the extraction time varied from 24-20 h, which was drastically reduced to 2-4 h in process 1 and process 2 and 3 h in process 3 and process 4 of the current study. Reduced processing time improves yield by minimizing collagen leaching during pre-treatment and collagen losses during extraction. In this study, yield, L-hydroxyproline, amino acid profile, and other parameters of the extracted gelatin were used to determine the efficiency of the extraction process. L-hydroxyproline is almost exclusively present in collagen and is therefore used to calculate the collagen and gelatin content.[24] L-hydroxyproline and proline directly influence the gel strength of gelatin whereas parameters of the extraction process influence both the yield of gelatin and its properties.[6]

Table 3: Comparison of current study with previous studies.

Parameters	Previous Studies		This Study		Conclusion
	Valcarcel[23]	Tümerkan[21]	Single Step extraction	Multiple step Extraction	
Number of processing steps	4	3	1	2	Reduced number of steps provided better control over the process and reduced losses
Chemicals used	Sodium hydroxide, sulphuric acid, citric acid	Sodium hydroxide, acetic acid	Acetic Acid	Sodium Chloride	Use of green solvent and elimination of harsh chemicals provided an environment friendly method of gelatin extraction
Processing Time	20 h	26 h	3 h	4 h	Reduced processing time minimizes chances of collagen leaching during extraction

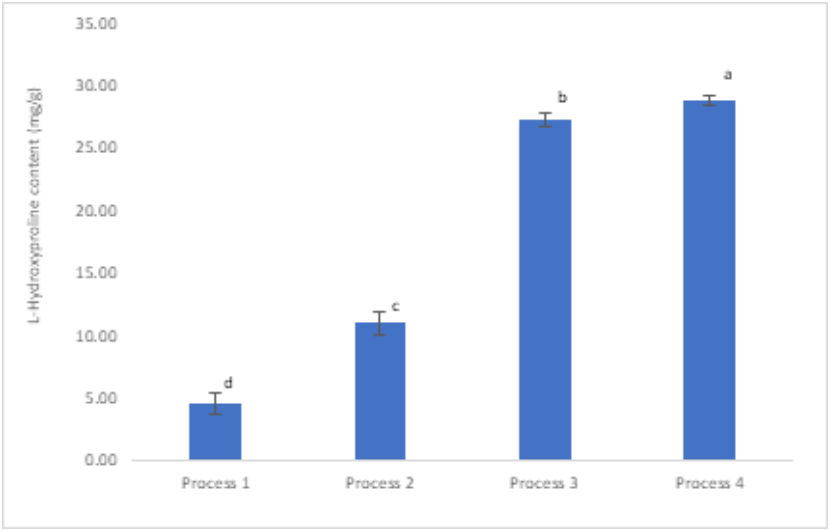


Fig. 5: L-Hydroxyproline content in gelatin extracted from pink perch skin and bones by process 1, process 2, process 3, and process 4

* Error bar indicates the standard deviations from three replications. Different lowercase letters indicate significant differences ($p<0.05$)

L- Hydroxyproline Content

Hydroxyproline is one of the most abundant amino acids in gelatin after glycine and proline. The hydroxyproline content is evaluated to determine the gelatin extraction yield, suggesting a successful extraction process. In this study, L-hydroxyproline content of the gelatin extracted from pink perch skin and bones biomass varied significantly with the process used for extraction ($p<0.05$). The highest L-hydroxyproline content was obtained

from extraction using acetic acid in process 4 (28.8±0.9 mg/g) followed by process 3 (27.3±0.9 mg/g). Significantly lower ($p<0.05$) L-hydroxyproline content was observed in gelatin obtained from process 2 (10.9±0.6 mg/g) and process 1 (4.6±0.4 mg/g) (Figure 5). This could be a result of different extraction methods resulting in varying degrees of purity in extracted gelatin. The higher L-Hydroxyproline content in gelatin extracted from process 3 and process 4 indicate higher purity levels, which may result in more desirable gelling properties such as, higher gel strength and viscosity. The L-hydroxyproline content of gelatin obtained with process 3 and process 4 were also significantly higher than earlier reported value of 7.63 mg/g from pink perch gelatin,[9] 6.3 mg/g from Tuna skin gelatin[21] and 6.2 mg/g from carp skin gelatin.[25] During gelling imino acids (L-hydroxyproline and proline) play an important role in the renaturation of gelatin subunits. Therefore, high levels of imino acids (L-hydroxyproline and proline) in gelatin implies higher gel strength and melting point.[9] The L-hydroxyproline content of the gelatin also reflect the gelatin yield.

Table 4: Gelatin yield, protein yield, and product yield of gelatin extracted from different green extraction processes

Green extraction processes	Product Yield (%)	Gelatin Yield (%)	Protein Yield (%)
Process 1	1.21±0.3^{b}	0.9±0.1^{b}	2.28±0.0^{b}
Process 2	1.51±0.2^{b}	2.7±0.1^{b}	5.46±0.0^{b}
Process 3	4.22±0.2^{a}	19.2±2.2^{a}	25.29±0.0^{a}
Process 4	3.92±0.5^{a}	18.8±3.0^{a}	22.91±0.0^{a}

*Values are represented as means ± the SD. Different lowercase letters within a column indicate a significant difference between the values of the column ($p \leq 0.05$)

Table 5: Proximate analysis of gelatin extracted from different green extraction processes

Gelatin	Moisture (%)	Fat (%)	Protein (%)	Ash (%)
Process 1	12.9±1.6^{a}	1.3±0.0^{a}	25.2±0.5^{d}	54.1±1.6^{a}
Process 2	12.7±0.6^{a}	1.0±0.0^{a}	48.1±1.2^{c}	35.3±1.5^{b}
Process 3	8.8±0.1b	1.0±0.0^{a}	79.6±0.8^{a}	7.4±0.6^{c}
Process 4	13.9±1.9^{a}	1.0±0.0^{a}	77.7±0.9^{b}	3.1±0.9^{d}

*Values are represented as means ± the SD. Different lowercase letters within a column indicate significant difference between the values of the column ($p \leq 0.05$)

Gelatin, Protein, and Product Yield

In the present study gelatin extraction efficiency of the four processes was compared on the basis of gelatin yield, protein yield and product yield. Protein yield indicates amount of protein extracted from the raw material while gelatin yield indicates the amount of gelatin extracted from the raw material. The highest gelatin yield from combined biomass of fresh skin and bones was obtained from process 3 and process 4 followed by process 2 and process 1 on the wet weight basis (Table 4). Significantly higher (19.2%) gelatin yield was obtained with the single-step extraction processes in comparison to gelatin yield (2.7%) from two-step extraction processes ($p<0.05$). Extraction with acetic acid also resulted in higher protein yield in comparison to the extraction done using NaCl pre-treatment. The yield of gelatin is significantly depended on extraction conditions.[6] Skin and bone biomass treated with acetic acid was subjected to higher ionic strength which could

have facilitated the extraction process by promoting swelling of collagen molecules resulting in higher yield.[25] The protein yield and gelatin yield of gelatin extracted using acetic acid were higher than the previously reported protein yield of 20.3% and gelatin yield of 15.3% reported for gelatin extraction from silver carp skin.[24] A comparison of L-hydroxyproline, protein content and gelatin yield provided valuable insight regarding effectiveness of the extraction process. Gelatin extracted using single-step extraction method, i.e., process 3 and process 4 had higher L-hydroxyproline content along with higher protein and gelatin yield indicating better extraction efficiency of the process. Gelatin yield was found to be directly proportional to the product yield and protein yield. Product yield is crucial for commercial production efficiency and financial viability. Product yield varies according to the collagen content, raw materials, and extraction conditions.[26] The highest product yield obtained (4.2 ± 0.2 %) on the wet-weight basis was in accordance with the previously reported study on gelatin extraction from pink perch skin gelatin (5.57%)[9] and pink perch bones gelatin (3.55%),[9] and higher than gelatin extracted from trout skin (1.56%).[23] The difference in gelatin yield between this study and previous reported studies could be explained by variations in the extraction methods. The multi-step extraction procedure may result in collagen leaching and subsequent losses during extraction process. Gelatin extraction from combined biomass of pink perch skin and bone using acetic acid indicated better results in terms of enhancement of both gelatin yield and recovery.

Proximate Composition

The proximate composition of pink perch skin and bone biomass gelatin (PG) extracted from different processes is shown in Table 5. The moisture content of lyophilized gelatin varied between 8.7 ± 0.1% -12.9 ± 1.6%. Since pink perch is a lean fish, therefore, low-fat content of less than 1.5% was observed in gelatin extracted from all processes. The protein content of gelatin obtained from process 3 and process 4 was in the same range as previously reported9 for pink perch gelatin (72.6%). Also, the protein content of process 3 and process 4 was significantly higher than process 1 and process 2 ($p<0.05$). A significant variation in the protein concentration of gelatin extracted from the different processes depicted the influence of extraction parameters on the protein content of gelatin ($p<0.05$). A similar pattern was observed in L-hydroxyproline content of extracted gelatins from different processes. A significantly high difference in ash content was also observed among gelatin obtained from different extraction processes ($p<0.05$). Process 1 and process 2 had significantly higher ash content in contrast to process 3 and process 4 ($p<0.05$), indicating that extraction parameters have a significant effect on the ash content of gelatin. Lower ash content in gelatin extracted from process 3 and process 4 can be explained by the fact that acetic acid helps in the reduction of ash content by removing alkali and alkali earth metals.[27,28] Calcium and phosphorus were the major minerals observed during mineral analysis of the skin and bones sample. The acetic acid possibly lowered the ash content by the formation of calcium and phosphorus salts which are water insoluble at neutral pH,[29] thus eliminating these minerals from the gelatin during extraction in processes 3 and process 4, which was not possible in extraction with NaCl i.e., process 1 and process 2. This was further validated by estimating the ash content of residue obtained in all processes (Table S2), which indicated higher ash content in residue obtained from acetic acid extraction than NaCl extraction. Though the ash content of gelatins extracted with acetic acid was slightly higher than the recommended levels (FSSAI, 2011), it can be further reduced to acceptable limits by washing the raw material with ethanol and further optimization of the extraction conditions. Other methods such as ion exchange can also be used to reduce the ash of the extracted gelatin.[30] These results suggested a significant effect of the extraction process on the proximate composition of the extracted gelatin, which was in agreement with the similar observation made in earlier studies.[31]

Amino Acid Composition

The amino acid composition of gelatin obtained from different processes is shown in Figure 6, Table S1. The result indicated that extraction parameters affected the amino acid composition of gelatin. Similar results have been reported by Tkaczewska[25] and Diaz-Calderon.[31] They observed that the amino acid composition of gelatin varied significantly with variations in process parameters. Since, approximately 60% of α-chains present in gelatin are composed of tripeptides with the formula of Gly-Pro-L-hyp.[29] Therefore, as expected glycine was one of the most abundant amino acids in all four processes, with the highest content being observed in

process 3. In processes 3 and 4, the content of imino acid (Proline + L-hydroxyproline) was observed to be higher than in processes 1 and 2. Result also suggested that glycine extraction was enhanced in process 2 due to an increase in extraction time as compared to process 1. Higher content of imino acid contributes towards better physical properties of gelatin and higher gel strength. L-hydroxyproline content obtained in processes 3 and 4 was at par with previously reported studies.[9] L-hydroxyproline significantly affects the thermal stability of triple helix structure by interacting with water molecules through its hydroxyl group.[33]

Fig. 6: Amino acid profile of pink perch gelatin obtained from process 1(P1), process 2 (P2), process 3 (P3), and process 4 (P4)

Conclusion

This study was done to assess the suitability of combined biomass of pink perch skin and bones as raw materials for gelatin extraction and to compare the efficiency of different green gelatin extraction processes utilizing this biomass. Proximate analysis of pink perch by-products suggested the presence of high protein concentration (>10%) in the combined biomass of skin and bone. SEM-EDX studies confirmed the absence of any heavy metal contamination. The microbiological assessment revealed the absence of any toxic microbes and that the investigated sample was within the specified limits of FSSAI. Hence, the sample was qualified for further processing into high-value products such as gelatin.

Gelatin extraction was carried out using four green extraction processes with variations in parameters which confirmed the effect of process parameters on gelatin yield and L-hydroxyproline content. Extraction performed with single-step extraction

using acetic acid recorded a higher gelatin and protein yield in comparison to the two-step extraction process involving NaCl pre-treatment. Further, the single-step extraction processes also produced gelatin with higher protein content, higher glycine, proline, and L-hydroxyproline content, and the lowest ash content. The ash content of the extracted gelatin can further be reduced to acceptable limits by washing with ethanol during sample preparation and further optimization of the extraction process. Thus, the acetic acid extraction process provides a single-step extraction method that does not require demineralization of raw material as acetic acid used during the extraction reduces the ash content significantly by forming insoluble residues. This process also reduces the sample preparation time making it simpler and easier to handle. The process also provides a cost-effective, environmentally friendly, and convenient way to valorize the surimi industry by-product.

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Conflicts of interest

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Disclosure Statement

NK and K designed the experiment. K conducted the experiment and wrote the manuscript. NK, KW, RS, and AK reviewed the manuscript.

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