Optimization of novel single-step gelatin extraction method using RSM model for valorization of surimi industry by-products

Khushboo

Amity University Uttar Pradesh, Noida <https://orcid.org/0000-0001-5768-7366>

Nutan Kaushik (✉ kaushikn2008@gmail.com)

Amity University Uttar Pradesh, Noida

Kristina Norne Widell

SINTEF Ocean, Norway

Rasa Slizyte

SINTEF Ocean, Norway

Asha Kumari

Amity University Uttar Pradesh, Noida <https://orcid.org/0000-0002-2554-5627>

Research Article

Keywords: Gelatin, Pink Perch, RSM, by-product utilization, Nemipterus japonicus, Rheological properties

Posted Date: January 14th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1261080/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

1 Optimization of novel single step gelatin extraction method using RSM model for
2 valorisation of surimi industry by-products

3 Khushboo^{1,2}, Nutan Kaushik¹, Kristina Norne Widell³, Rasa Slizyte³, Asha Kumari^{1,2}

4 ¹ Amity Food and Agriculture Foundation, Amity University Uttar Pradesh, Noida 201313, India

5 ² Amity Institute of Food Technology, Amity University Uttar Pradesh, Noida 201313, India

6 ³ SINTEF Ocean, Dep. Seafood Technology, 7465, Trondheim, Norway

7

8

9

10

11 Corresponding Author

12 Dr. Nutan Kaushik

13 Amity Food and Agriculture Foundation

14 Amity University Uttar Pradesh

15 Noida 201313

16 +91 9811392249

17 Nkaushik5@amity.edu

18 kaushikn2008@gmail.com

19

Abstract

Surimi industry produces large quantity of by-products as a combination of skin, bones, and scale, which due to technical difficulty in separation, are being currently utilized for production of low- value products such as biofertilizers and fish feed. Present paper focuses on utilization of combined skin, bones, and scale from Pink Perch (*Nemipterus japonicus*) obtained from surimi industry for gelatin extraction using single step process. Single step extraction method with acetic acid and water was optimized using Response Surface Methodology (RSM) to maximize yield and gel strength so that the process can be applied for sustainable utilization. Parameters such as pH (A), extraction temperature (B) and extraction time (C) with respect to yield and L-hydroxyproline content were optimized. Highest gelatin yield was obtained at pH 3, 75°C extraction temperature, and 30 min extraction time. Gelatin yield and L-hydroxyproline content under optimum condition were 16.2% and 41.62 mg.g⁻¹. The chemical composition, functional, rheological, and structural properties of gelatin were examined and compared with commercial bovine gelatin. Gelatin thus obtained at optimized condition exhibited high gel strength (793g) and higher imino acid content (18.1%) than bovine gelatin. FTIR spectra depicted high similarities between both gelatin sample. Thus, the optimized method can be utilized for gelatin extraction from Pink Perch by-products for development of high value products such as food application.

Keywords: Gelatin, Pink Perch, RSM, by-product utilization, *Nemipterus japonicus*, Rheological properties

1 Introduction

Fish are the on the major sources of protein across the globe, leading to the expansion of fish production from 86.9 MT in year 1986 to 96.4 MT in year 2018 (FAO, 2020), which has resulted in an increase in the quantity of by-products generated. Approximately 40% of the harvested fish is consumed during the processing while remaining nearly 60% of the fish are not utilized to its full potential (Elavarasan et al., 2017). These by-products predominantly include head and viscera, skin and bones trimmings, livers, roes etc. and they are either discarded or utilized in production of low value product such as fish feed or fertilizers. This has led to serious ecological threat due to high organic load and economical losses due to underutilization and loss of nutritionally valuable materials. These by-products are rich source of commercially valuable biomolecules such as proteins, gelatin, omega-3 polyunsaturated fatty acids, vitamins (A, D, E, K), enzymes, bioactive peptides, amino acids etc. Therefore, currently many studies are being conducted towards utilization of these by-products to exploit their full potential as high value products is becoming global focus.

Gelatin is a protein based functional biopolymer produced from irreversible hydrolysis of collagen molecules present in skin, bones, and scale of animals. It has a vast application in the field of food, pharmaceuticals, photography, cosmetics, and biomedical application due to its functional properties (Kumar et al., 2017). Conventionally, gelatin from mammalian source is being utilized commercially. However, due to association of risk factors such as probability of contraction of pathogenic infections such BSE (Bovine Spongiform Encephalopathy), prions and foot and mouth disease and religious constraints, fish gelatin are now being considered as suitable alternatives (Koli et al., 2012; Jin et al., 2019). In addition, fish derived gelatin are also promising source of bioactive compounds exhibiting, antioxidant, anticarcinogenic properties (Tkaczewska et al., 2018; Mirzapour-Kouhdasht et al., 2020).

Though many studies have been conducted for extraction of gelatin from various fish by-products (Martins et al., 2018; Zhang et al., 2020; Tan et al., 2019, Mi et al., 2019), very few studies are available for combined biomass of skin, bones, and scales utilization. This study utilizes combined biomass of skin, bones, and scale from surimi industry particularly from Pink Perch, for extraction of good quality gelatin for utilization in food product development. Pink Perch is a highly important commercial fish utilized in India for production of surimi due to its

low cost, white and lean meat (Singh et al., 2005). In surimi production, a combination of skin, bones and scale are produced as mixed by-products. The production process makes it impossible to create separate by-products streams. Since conventional methods of production of high value products such as gelatin involves use of skin and bones separately, the by-products from surimi processing are commonly used for production of biofertilizers, fish feed or discarded due to technical difficulty in separation of skin, bones, and scale combination industrially. Therefore, development of technology for utilization of combined biomass of Pink Perch by-products for gelatin production will provide eco-friendly and economically viable solution for industries. Utilization of organic acid for extraction of gelatin is a cost-effective method. Additionally, the method is environmentally sustainable, and the final products does not contain any toxic chemical.

Therefore, objective of this study was to optimize gelatin extraction from combined biomass of skin, bones, and scales from Pink Perch using Response Surface Methodology (RSM) to increase the yield and properties of extracted gelatin. Subsequently, characterize the optimized gelatin for its rheological properties, functional properties and structural properties and compare with commercial bovine gelatin (BG).

2 Experimental

2.1 Materials

The Pink Perch by-products (combination of skin, bones, and scales) were procured from Ulka Seafood Pvt. Ltd, Mumbai in frozen condition (-20°C) and stored at -20°C until further use. Gelatin from bovine source (Himedia) was used as control sample. All the reagent used were of analytical grade.

2.2 Measurement of Swelling Efficiency

Swelling efficiency of Pink Perch by-products was analyzed according to method described by Mirzapour-Kouhdasht et al., (2020). The swelling process was carried out at various pH values (1-14) maintained using 0.1N HCl and 0.1N NaOH. Mixture of skin, bones and scale was thawed at room temperature (25°C) for 30 minutes and weighed. The thawed mixture was then soaked in various solutions of pH value ranging from 1-14 in ratio of 1:10 (w/v) and incubated at room

99 temperature (25°C) for 3 hours. The mixture was neutralized and washed with distilled water
 100 before taking final weight. The swelling efficiency was calculated as-

101
$$SE (\%) = \frac{\text{weight of sample after swelling}}{\text{weight of sample before swelling}} \times 100$$

102 2.3 Optimization of gelatin extraction

103 Response Surface Methodology (RSM) was utilized to analyze the effects of independent
 104 variable i.e., pH, time, and temperature of gelatin extraction treatment in response to dependent
 105 variable i.e., yield and L-hydroxyproline content of the gelatin. RSM is mathematical modeling
 106 and statistical tool that interrelates extraction conditions and product attributes by establishing
 107 regression equation. Design Expert Version 12.0 was used to obtained design experiment based
 108 on Box-Behnken Design. Each independent variable varied at three levels -1, 0, +1 (Table 1) and
 109 17 runs were obtained with 5 center points to minimize the error (Table 2).

110 Table 1

111 Experimental design of independent variables in Box-Behnken Design for gelatin extraction
 112 from Pink Perch by-products.

Independent Variable	Symbols	Range		
		-1	0	+1
pH	A	3	6	9
Temperature(°C)	B	25	50	75
Time (min)	C	30	135	240

113

114 Table 2:

115 17 experimental design points used for Pink Perch by-product gelatin extraction

Run	Independent Variables		
	pH (A)	Temperature (°C) (B)	Time (min) (C)
1	3	25	135
2	9	50	30

3	6	75	30
4	3	50	240
5	6	50	135
6	3	50	30
7	9	50	240
8	6	50	135
9	6	50	135
10	6	75	240
11	9	25	135
12	3	75	135
13	6	25	240
14	6	25	30
15	6	50	135
16	6	50	135
17	9	75	135

116

117 2.3.1 Process for gelatin extraction

118 Figure 1 shows the process of gelatin extraction, from whole fish to freeze-dried gelatin.
119 Homogenous mass of Pink Perch by-products (25 g) was thawed and cut into small pieces of size
120 1cm². The cut pieces were mixed with distilled water in a ratio of 1:3 (w/v) and stirred at room
121 temperature (25°C) for 10 minutes (Figure 1). Extraction process was carried out by subjecting
122 the mixture to 17 combinations of pH (3-9), temperature (25-75), time (30-240 minutes)
123 provided by RSM software. Glacial acetic acid and 4M NaOH were used to adjust pH of the
124 aqueous solution. pH of the reaction mixture was neutralized to pH (6-6.5) after reaction using
125 glacial acetic acid or 4M NaOH. Afterwards the non-solubilized material was filtered. The
126 permeate solution containing solubilized gelatin was freeze dried.

127

128

Fig 1. Schematic diagram of optimization of gelatin extraction from Pink Perch by-products

2.3.2 Yield

Yield of extracted gelatin was calculated as per using following equations -

$$\text{Yield (\%)} = 100 \times \frac{\text{Weight of lyophilised gelatin (g)}}{\text{Weight of initial fresh sample (g)}}$$

2.3.3 L- Hydroxyproline content

L- Hydroxyproline (L- Hyp) content was estimated using the method of Koli et al., (2012). The sample was hydrolyzed using 12 M HCl for 3 hours at 110°C and filtered. The filtrate was neutralized using 1M NaOH to a pH of 6.0-6.5. The neutralized sample (0.1 mL) was mixed with 0.2 mL of isopropanol oxidant solution (0.1 mL of the mixture of 7% chloramine -T and

acetate/citrate buffer pH 6 in a ratio of 1:4; v/v). Enhlrich's Reagent (1.3 mL) was added to the mixture and incubated at 60°C for 30 minutes. Later it was cooled for 2-3 minutes, and volume was made up to 5 mL using isopropanol. Absorbance was measured at 558 nm using LMSPUV1000B, UV-VIS spectrophotometer (Labman Scientific Instruments Pvt.Ltd., India). L-Hydroxyproline solution ranging from 0.01 mg.g⁻¹ to 0.1 mg.g⁻¹ was used as standard. Hydroxyproline content was expressed in mg.g⁻¹

2.4 Characterization of the Gelatin

2.4.1 Proximate Analysis

Proximate composition of gelatin was determined as per standard methods of AOAC. For moisture content determination 1g of gelatin was oven dried at 105°C for 24 hours and final weight was recorded after cooling down the dried sample in desiccator. Ash content was estimated by keeping the moisture free sample in muffle furnace at 600°C for 24 hours for dry ashing. Fat content was analyzed using Soxhlet method. Initial and final weights were recorded. Total nitrogen content (N) was estimated by CHN-S/N elemental analyzer 1106 (Costech Instruments ECS 4010 CHNSO Analysator). Protein content was subsequently calculated using factor of 5.55 (Kurl, 2019).

2.4.2 Gel Strength and Texture Profile Analysis

Gelatin (6.67g) was mixed with 100 mL distilled water using a water bath at 60°C until completely dissolved. 10 mL of gelatin solution was transferred to silicone tray and refrigerated at 4°C for 16-18 hours for maturation. Gel strength measurements was carried out using TA- XT Plus texture analyzer (Stable Micro Systems, Surrey, UK) attached with a 5KN load cell. The force required to penetrate cylindrical flat bottom probe (12.7mm diameter) to 4mm in gelatin sample at a speed of 1mm/s was considered as a gel strength in g.

For Texture profile analysis (TPA) gel sample were demolded from silicone tray. TA-XT Plus texture analyzer (Stable Micro Systems, Surrey, UK) equipped with load cell of 50 kg was used to conduct TPA. A 75 mm diameter probe was used to compress gelatin gel twice to 40% of its original height as an imitation of chewing process. The time duration between first and second compression was 5 sec. Textural parameters such as hardness (g), cohesiveness, adhesiveness

(g.sec), springiness and chewiness were calculated by Exponent Connect software provided along with the instrument.

2.4.3 Rheological Measurements

For rheological measurement 6.67% gelatin solution was prepared to 60°C until completely dissolved. Mixture was matured at 4°C for 16-18 hours. The elastic modulus (G') and loss modulus (G'') was recorded for gelatin gels using Anton Paar Germany GmbH, MCR 52 modular compact rheometer. Strain sweep of gelatin gels was conducted at a frequency of 1Hz at 5°C for 0.1% to 10% strain to determine the linear viscoelastic region of sample. The temperature sweep test was conducted from 5°C to 35°C and 35°C to 5°C. During the test a scan rate of 1°C/min, 1Hz frequency and 3Pa stress was maintained. The temperature corresponding to crossover point of G' and G'' during heating and cooling of gelatin sample were defined as melting and gelling points respectively. Frequency sweep test was done to analyze stability and structure of gelatin gel. The test was conducted at 5°C between 0.01 and 100 Hz and 3 Pa stress. The time sweep test was performed for 30 minutes at a temperature of 5° C at a frequency of 1Hz and 200 pa stress.

2.4.4 Water Holding and Fat Binding Capacity

Water holding and fat binding capacity was determined as per the method of Jin et al., (2019) with slight modification. Estimation of water holding capacity was conducted by adding 100 mg of the lyophilized gelatin powder to 1000 μ L of distilled water and allowing to stand for 1 hour at ambient temperature (25°C). The suspension was centrifuged at 6000 $\times$ g rpm for 20 minutes at 25°C. The upper layer was decanted by allowing the tube to stand at 45°C for 30 minutes. Weight of residue was noted. Fat binding capacity was calculated by replacing distilled water with 1000 μ L sunflower oil.

2.4.5 Foaming Capacity and Foam Stability

Foam expansion and foam stability was determined as per method of Dara et al., (2020) with slight modification. Gelatin solution of different concentration (0.1%, 0.5%, 1%) was prepared and homogenized for 2 minutes at room temperature (25°C). Aliquot was immediately transferred to 50 mL measuring cylinder and volume of whipped sample was recorded. The aliquot was allowed to stand for 30 minutes at room temperature and volume was recorded.

196 The foam expansion and foam stability were calculated as follows-

197
$$\text{Foam Expansion}(\%) = \frac{V_t - V_o}{V_o} \times 100$$

198
$$\text{Foam Stability} (\%) = \frac{V_T - V_o}{V_o} \times 100$$

199

200 Where-

201 V_o was total volume before homogenization

202 V_t was total volume after homogenization

203 V_T was total volume after standing for 30 minutes

204 2.4.6 Emulsion Activity Index and Emulsion Stability Index

205 The emulsion activity and emulsion stability were evaluated as per method of Dara et al., (2020).

206 Gelatin solution of various concentration (0.1%, 0.5%, 1%) were prepared. 30 mL of gelatin
207 solution was homogenized with 10 mL of sunflower oil for 1 minute. A 50 μ L aliquot was drawn
208 from the bottom of the emulsion at 0 minute and 10 minutes and diluted with 5 mL of 0.1% of
209 SDS solution. Absorbance of the solution was read at 500 nm using LMSPUV1000B, UV-VIS
210 spectrophotometer (Labman Scientific Instruments Pvt.Ltd., India) immediately after 0 minute
211 and 10 minutes.

212 Emulsion Activity Index (EAI) and Emulsion Stability Index (ESI) were calculated using
213 following equation-

214
$$\text{EAI (m}^2 \cdot \text{g}^{-1}) = \frac{2 \times 2.303 \times A_{500\text{nm}}}{0.25 \times \text{gelatin weight(g)}}$$

215

216
$$\text{ESI (min)} = \frac{A_o \times \Delta t}{\Delta A}$$

217

218 Where, ΔA = Absorbance at 0 min (A_o) – Absorbance after 10 min (A_{10}) and Δt = 10 min

219 2.4.7 Color

220 Color of gelatin was estimated by NS810 portable spectrophotometer (Shenzhen Threenh
221 Technology Co., Ltd., China) by employing CIE color system for evaluating L* (Lightness), a*
222 (redness/greenness), b* (yellowness/greenness) values. h* (hue) and C* (Chroma) were also
223 obtained using color analyzer.

224 2.4.8 Amino Acid Analysis

225 The amino acid composition of gelatin was determined by using High Performance Liquid
226 Chromatography (HPLC) as per the method of Osnes and Mohr, (1985) with slight modification.
227 50 mg of gelatin were weighed in a screw cap bottle and mixed with 2 mL of 6M HCl. The tubes
228 were incubated for 24 hours at 110°C. After completion of hydrolysis pH of the mixture was
229 neutralized and it was filtered using Whatman glass microfiber filters (55mm). Volume of the
230 filtrate was made-up up to 5mL using milli-Q water. The HPLC system (Agilent Infinity 1260,
231 Agilent Technologies) was coupled to an on-line-post-column derivatization module (Pinnacle
232 PCX, Pickering laboratories, Mountain View, CA, USA) using ion exchange column (4.6 x 110
233 mm, 5 mm) and OPA and FMOC as derivatizing reagent. Standard curves measured with amino
234 acid standards were used to quantify standard amino acids.

235 2.4.9 FTIR Analysis

236 FT-IR spectra of gelatin was obtained by using Nicolet FTIR 6700 (Thermo Fisher Scientific,
237 US). 5mg of dried sample was mixed with potassium bromide to form homogeneous powder.
238 The powder was mounted on the equipment and converted into pellets after subjecting to
239 pressure applied by hydraulic press. The pressed pellets were then analyzed in the region of
240 4000-500cm⁻¹ with 4cm⁻¹ resolution to determine the presence to of various functional groups.
241 Background corrections were carried out before analysis of samples.

242 2.4.10 SEM Analysis

243 Microstructure of 6.67% gelatin gels was visualized using Zeiss (MA EVO -18 Special Edition)
244 scanning electron microscopy. 2-3 mm thick gelatin samples were initially fixed with
245 glutaraldehyde (2.5%, v/v) in presence of 0.2 M phosphate buffer of 7.2 pH for a time period of
246 12 h. These samples were subsequently rinsed with distilled water and dehydrated using ethanol.

Dehydrated samples were mounted on bronze stub and coated with gold and palladium in a process called gold sputtering. This process was carried for 4 minutes. And samples were observed at an acceleration voltage of 20kv.

2.5 Statistical Analysis

All the experiments were conducted in triplicates. Mean and standard deviation were calculated using Microsoft Office Excel 2016. Statistical analysis of data was carried out using the IBM SPSS (Version 26.0). The data presented in the study was analyzed by one-way analysis of variance (ANOVA) The Duncan's multiple range test was used to determine the significance of mean differences. The significant level in the present study was set at $p < 0.05$

3 Results and discussion

3.1 Measurement of Swelling Efficiency

Swelling efficiency of by-products is a very crucial factor in determining intensity of pre-treatment required for gelatin production from fish. Swelling efficiency is primarily affected by absorption caused by collagen present in skin, bones, and scale. High swelling efficiency indicates high interaction of collagen molecules with the external medium resulting in less resistance and lower interference from surrounding biomolecules during absorption of water or other external medium (Mirzapour-Kouhdasht et al., 2020). Therefore, a higher swelling efficiency implies higher possible yield of gelatin. In this study higher swelling efficiency was observed at acidic ($\text{pH} < 3$) and alkali ($\text{pH} > 10$) pH in comparison to neutral pH conditions (Figure 2). A sharp decline in swelling property was being observed at highly alkali and acidic condition which can be due to fragmentation of protein into smaller peptides.

Fig. 2. The effect of pH on swelling efficiency of by-products of Pink Perch

3.1.1 Optimization of gelatin extraction

Quality of gelatin depends on nature, source of raw material, and yield, but also on extraction parameters. Therefore, optimization of extraction parameters is required to maximize the efficiency of extraction process. In this study, RSM was employed to obtain the optimum condition for gelatin extraction from Pink Perch by-products.

Box-Behnken Design with three independent variables (pH, temperature, and time) was followed to study yield and L-hydroxyproline as response variable. Regression analysis was performed for all responses. Models obtained by RSM for yield and L-hydroxyproline content of gelatin was statistically significant. High R^2 value was obtained for both the models.

3.1.1.1 Yield

From commercial point of view, obtaining maximum yield is a prime objective for any extraction method. In this study, gelatin yield obtained at 17 combinations provided by RSM-BBD was in range of 0.03% - 13.06% (Figure 3). A quadratic relationship was observed between

extraction parameters and gelatin yield. Regression equation for prediction of maximum yield obtained from RSM is provided at equation 1. The model was found to be significant with p-value < 0.001. A high p-value of lack of fit implies that quadratic model was adequate. The results from ANOVA suggested that gelatin yield was significantly influenced by all the independent variables i.e., pH (A), temperature (B) and time (C) and their interactions (p<0.001). R² value (0.99) obtained from analysis of variance signifies that model was capable of sufficiently explaining the system. Optimum condition for extraction of maximum gelatin yield (16.2%) obtained from RSM was observed to be pH 3, time 30 minutes and temperature 75°C. Verification of predicted gelatin yield obtained from model was done by conducting gelatin extraction at optimum condition in triplicates. Experimental yield (16.2%) obtained after performing extraction at optimum condition was close to predicted value (16.9%) (Table 3). Gelatin yield obtained in this study was higher than previous reported studies such Mafazah et al., (2018) reported a yield of 8.4%- 12.49% from Yellowfin Tuna fish skin and Hue et al., (2017) reported yield in the range of 10.2- 13.8% from European perch and Volga Pikeperch skins.

$$\text{Yield} = 24.20223 - 6.22941A + 0.107592B - 0.063355C - 0.008217AB + 0.008698AC - 0.000162BC + 0.353292A^2 + 0.000615B^2 + 0.000040C^2$$

Equation 1

Where, A= pH, B= Temperature (°C) and C= Time (min)

Fig 3: Yield of gelatin from Pink Perch by-products obtained at different RSM combinations and optimum condition. Condition for extractions of 1-17 is presented in table 2 and for optimum condition is presented in table 3

Table 3
Predicted and experimental values of percentage yield and L-Hydroxyproline content (L-Hyp) of gelatin from Pink Perch by-products at optimized condition

pH (A)	Temperature (°C) (B)	Time (min) (C)	Predicted Value (Yield %)	Optimized Value (Yield %)	Predicted Value (L- Hyp, mg.g ⁻¹)	Optimized Value (L- Hyp, mg.g ⁻¹)
3	75	30	16.9	16.2	39.99	41.62

3.1.1.2 L-Hydroxyproline

A linear relationship was observed between L-hydroxyproline content and independent variables (pH, extraction temperature and extraction time) from RSM. L hydroxyproline content obtained at 17 RSM combination ranged from 2.78 -43.77 mg.g⁻¹ (Figure 4). Regression equation 2 was

obtained to predict L-hydroxyproline content using this model. Analysis of variance of response surface model for L-hydroxyproline suggested that the model was highly significant with p-value <0.01. It was observed that L-hydroxyproline content of gelatin was significantly influenced by the independent variables i.e., pH, and temperature with p<0.05. However, interaction of various extraction factor did not produce any significant effect on L-hydroxyproline content. Verification of predicted value was conducted in triplicates at optimum condition (pH 3, temperature 75°C and time 30 minutes). Predicted value of L-hydroxyproline content based on RSM analysis (39.99 mg.g⁻¹) was close to the experimental value of gelatin extracted from (41.62 mg.g⁻¹) (table 3). L-hydroxyproline of Pink Perch by-products gelatin obtained at optimum condition was significantly higher than control bovine gelatin (34.97 mg.g⁻¹) (p<0.05).

$$\text{L-Hydroxyproline} = 0.360768 - 0.127475A + 0.054344B - 0.001946C \quad \text{Equation 2}$$

Where, A= pH, B= Temperature (°C) and C= Time (min)

Fig. 4. L-hydroxyproline content of gelatin from Pink Perch by-products obtained at different RSM combinations. Condition for extractions of 1-17 is presented in table 2 and for optimum condition is presented in table 3.

330 3.1.1.3 Response surface plots

331 A three-dimensional response surface plots of effects of independent variables (pH, extraction
 332 temperature and extraction time) on dependent variable (yield and L-hydroxyproline content) are
 333 shown in Figure 5 & 6. The graphs are plotted as a function of two factors at a time and keeping
 334 the third factor fixed at middle level. From plots it was observed that as the level value of
 335 extraction time and pH decreased, gelatin yield and L-hydroxyproline content increased and
 336 increase in extraction temperature led to increase in gelatin yield and L-hydroxyproline content.

Yield
 Design Points:
 ● Above Surface
 ○ Below Surface
 0.03 13.06

Yield = 6.28
 Std # 1 Run # 11
 X1 = A: pH = 3
 X2 = B: Temperature = 25

Actual Factor
 C: Time = 135

(a)

Yield
 Design Points:
 ● Above Surface
 ○ Below Surface
 0.03 13.06

Yield = 13.06
 Std # 5 Run # 4
 X1 = A: pH = 3
 X2 = C: Time = 30

Actual Factor
 B: Temperature = 50

(b)

Yield
 Design Points:
 ● Above Surface
 ○ Below Surface
 0.03 13.06

Yield = 0.98
 Std # 9 Run # 16
 X1 = B: Temperature = 25
 X2 = C: Time = 30

Actual Factor
 A: pH = 6

(c)

Fig. 5. 3- D surface graph of gelatin yield from Pink Perch by-products as a function of (a) temperature vs pH, (b) time vs pH, (c) time vs temperature

(a)

(b)

(c)

Fig. 6. 3-D Graph of L-Hydroxyproline content of gelatin from Pink Perch by-products as a function of (a) pH vs time, (b) time vs temperature and (c) pH vs temperature

3.2 Proximate composition of the extracted gelatin

Gelatin obtained at optimized condition was observed to have moisture content of $4.79 \pm 1.45\%$ which was considerably lower than bovine gelatin which has moisture content up to $12.11 \pm 0.02\%$ (Table 4). Moisture content of Pink Perch by-products gelatin (PG) was in accordance with the recommended limit prescribed for edible gelatin that is less than 15% (FSSAI, 2011). Protein content of optimized gelatin ($92.06 \pm 0.99\%$) was higher than the bovine gelatin (BG) ($83.44 \pm 1.14\%$). Optimized gelatin has $2.56 \pm 0.10\%$ ash content in comparison of $0.89 \pm 0.38\%$ of ash content in bovine gelatin, which was within the recommended limits for edible gelatin that is less than 3% (FSSAI, 2011). Since Pink Perch is a lean fish a relatively lower fat content was observed in optimized gelatin ($0.13 \pm 0.02\%$) as compared to bovine gelatin ($3.36 \pm 0.17\%$). The fat content of Pink Perch gelatin was less than 1%, suggesting its potential application in food products. Based on results obtained it can be concluded that proximate content of gelatin is influenced by methods of extraction employed.

Table 4

Proximate analysis of Pink Perch by-products gelatin from optimized condition and bovine gelatin

Gelatin	Ash (%)	Moisture (%)	Fat (%)	Protein (%)
Bovine Gelatin	0.89 ± 0.38	12.11 ± 0.02	3.36 ± 0.17	83.44 ± 1.14
Optimized Gelatin	2.56 ± 0.10	4.79 ± 1.45	0.13 ± 0.02	92.06 ± 0.99

Values are means $\pm$ standard deviation (n=3)

3.3 Gel Strength

Gel strength is one of defining characteristics of gelatin and hence it also acts as a quality indicator. On the basis of gel strength gelatins are classified into 3 categories low bloom (< 150 g), medium bloom ($150\text{--}220$ g) and high bloom ($220\text{--}300$ g). In this study high bloom gelatin was obtained from PG with gel strength of 793g, significantly higher than gel strength of medium bloom BG (167g) (Table 5). Gel strength of gelatin are influenced by various intrinsic and extrinsic factors such as extraction parameters, amino acid profile, imino acid content and

band intensities of β - and γ - chains. Gelatin forms a thermally reversible gel networks majorly stabilized by hydrogen bonds. These hydrogen bond formations are predominantly contributed by L-hydroxyproline via interaction with carbonyl group and bonding with water molecules. This was also demonstrated in this study where high gel strength of Pink Perch by-products gelatin was coincidental with high L- hydroxyproline content. Different studies have reported different gel strength of gelatin obtained from different fish such as yellow fin tune (426 g) (Cho et al., 2005), silver carp skin gelatin (770g) (Boran et al., 2010).

TPA tests of PG and control BG was done at 6.67% (w/v) concentration (Table 5). Generally, good gelatins are characterized by higher gel strength and TPA values (for hardness, cohesiveness, adhesiveness, springiness, and chewiness) (Yang et al., 2007). Hardness is defined as measure of strength of internal gel structure when subjected to compression. It is also referred as the force required to attain a certain deformation in gel structure. Hardness of PG (11.25 ± 1.11 N) was significantly higher than BG (5.15 ± 0.28 N) ($p < 0.05$). Cohesiveness is a measure of strength required to disrupt the internal structure of gel before absolute deformation. It is also an indicator of extent to which gelatin is able to withstand second compression in relation to first compression. Among both the samples tested PG showed significantly higher cohesiveness value (0.89 ± 0.01) than BG (0.78 ± 0.01) ($p < 0.05$). Adhesiveness in food products represents the amount of negative force area required for the first bite. It can be used to predict the degree of work necessary to overcome adhesion between food particle and teeth. PG showed similar adhesiveness value to BG. Springiness is defined as height recovered by food during timelapse between end of first bite and beginning of second bite. It is also called elasticity. Higher springiness in PG is indicative of formation of large pieces during TPA compression and lower springiness in BG might have resulted for formation of smaller pieces during TPA compression. Chewiness is defined as the product of hardness, cohesiveness, and springiness. It is a representative of energy required to masticate food product before swallowing. Chewiness value of PG was observed to be higher than BG. From TPA of both the sample it was evident that textural properties of PG were comparable to control BG.

Table 5:

Textural properties of Pink Perch by-products gelatin (PG) and bovine gelatin (BG)

Textural Parameter	PG	BG
Gel Strength(g)	793.01 ± 0.99	167 ± 1.00
Hardness (N)	11.25 ± 1.11	5.15 ± 0.28
Adhesiveness (g.sec)	-17.08 ± 3.78	-11.72 ± 1.05
Springiness	0.91 ± 0.01	0.78 ± 0.01
Cohesiveness	0.89 ± 0.01	0.75 ± 0.01
Chewiness	8.16 ± 0.94	3.89 ± 0.21

Values are means ± standard deviation (n=3)

3.4 Rheological Measurements

Figure 7 illustrates results from strain sweep test of PG and BG at 5°C. Strain sweep test was performed to estimate viscoelastic region of gelatin gel at 5°C. Results indicated that both the gelatin sample had higher storage modulus as compared to loss modulus, which leads to formation of stronger three-dimensional structure in gelatin. However, PG storage modulus was even higher than BG suggesting better gelling properties.

413
 414 Fig 7: Storage modulus (G') (square symbols) and loss modulus (G'') (triangle symbols) as a
 415 function of strain during a strain sweep at cooling for Pink Perch by-products gelatin (Red) and
 416 bovine gelatin (Blue)

417
 418 Cooling scan of PG and BG depicted a similar behavior of visco-elastic property (Figure 8). Both
 419 storage (G') and loss (G'') modulus for PG and BG increased linearly, followed by sharp
 420 increase in the curve between temperature of $16^{\circ}\text{--}5^{\circ}\text{C}$, $16^{\circ}\text{--}5^{\circ}\text{C}$ and $19^{\circ}\text{--}15^{\circ}$, $19^{\circ}\text{--}15^{\circ}\text{C}$
 421 respectively. Steep rise of G' and G'' curves are observed during of gelation due to thermal
 422 transitioning during which gel networks are formed rapidly. Gelling temperature is determined
 423 by cross-over point of G' and G'' curve in cooling scan of gelatin. Gelling temperature for PG
 424 and BG were 18.16°C and 20.1°C respectively.

425 Similarly, during heating scan both the gelatins indicated a steep rise in G' and G'' in
 426 temperature range of $13^{\circ}\text{--}25^{\circ}\text{C}$, $13^{\circ}\text{--}25^{\circ}\text{C}$ and $13^{\circ}\text{--}25^{\circ}\text{C}$, $15^{\circ}\text{--}25^{\circ}\text{C}$ respectively (Figure 8). A
 427 sharp decrease of G' and G'' in heating scan indicated the period during which gelatin is

undergoing thermal transition to form solution from gel state. Melting temperature is also taken as the cross-over point of G' and G'' curve in heating scan of gelatin. Melting temperature as observed from Figure 8 (c) and (d) for PG and BG were 24.83°C and 24.49°C respectively. Similar results were reported by Abdelhedi et al., (2017) for gelling (19.4°C) and melting temperature (25.3°C) of gelatin from black-barred halfbeak skin. He also mentioned that typical range for gelling and melting temperature of fish gelatin is 8 to 25°C and 11 to 28°C .

(a)

(b)

(c)

(d)

Fig 8: (a) Storage modulus (G') (black symbols) and loss modulus (G'') (red symbols) as a function of temperature during a temperature sweep at cooling for (a) Pink Perch by-products gelatin, (b) bovine gelatin and followed by heating for (c) Pink Perch by-products gelatin, (d) bovine gelatin (both at $1^{\circ}\text{C min}^{-1}$)

Study of storage modulus and loss modulus as a function of frequency is conducted to predict the possible behavior of gelatin during storage and thereby its applicability in food products. The frequency sweep test of PG and BG indicated little to no-effect of frequency on G' and G'' suggesting formation of a strong gel network under the test conditions (Figure 9). Similar results were obtained by Boran et al., (2010) and Tan et al., (2019) with silver carp skin and tilapia skin gelatin respectively.

Fig 9: Frequency-dependent changes in storage modulus (G') (circle symbols) and loss modulus (G'') (triangle symbols) at 5°C for Pink Perch by-products gelatin (Red) and bovine gelatin (blue)

Figure 10 illustrates time sweep test of PG and BG at 5°C . Storage modulus (G') of both gelatin samples was higher than its corresponding loss modulus (G'') over the testing range indicating

that the gelatin had reached its gelling point at 5°C. G' of PG was time independent whereas G' of bovine gelatin slightly decreased with time suggesting time dependency of BG in response to applied stress. Behavior of gelatin as a function of time are majorly influenced by gelation kinetics which are in turn affected by molecular weight and amino acid profile of gelatin (Tan et al., 2019). High G' of PG was in agreement with its good gel strength.

Fig 10: Time-dependent changes in storage modulus (G') (square symbols) and loss modulus (G'') (diamond symbols) at 5°C for Pink Perch by-products gelatin (Red) and bovine gelatin (blue)

3.5 Water Holding and Fat Binding Capacity

Water holding and fat binding capacity can be defined as the ability of a system to imbibe water/fat and hold it against gravitational pull. Water Holding Capacity (WHC) of PG ($207.27 \pm 2.61\%$) was significantly higher than BG ($78.6 \pm 1.64\%$) ($p < 0.05$) (Figure 11). The result obtained in the study was similar to WHC of European Perch ($227 \pm 3\%$) and Volga Pikeperch ($235 \pm 4\%$) (Hue et al., 2017) skin gelatins. There are multiple factors affecting WHC of gelatin such as hydrophobic amino acid content and amount of voids and pores present in micro-structure of gelatin. WHC is an important functional property responsible for determining drip losses and imparting juiciness to meat product during cooking/thawing.

Fat Binding Capacity (FBC) of gelatin can be attributed to presence of hydrophobic amino acids and extent of non-covalent bonds influencing protein-lipid interactions. FBC of PG (347.01±4.02%) was observed to be significantly higher than BG (177.06±1.86%) ($p<0.05$) (Figure 11).

These interfacial traits of gelatin influence textural properties of food by interacting with water, oil along with other components and hence determining applicability of gelatin in food products.

Fig. 11. Percentage of Water holding capacity (WHC) and percentage fat binding capacity (FBC) of bovine gelatin (BG) and Pink Perch by-products gelatin (PG). Values presented as average of three measurements with SD.

3.6 Foaming capacity and foaming stability

In the present study foaming capacity and foaming stability of PG and BG was examined over different concentration. Foaming capacity was observed to be in range of 8.89%- 15.6% for PG and 7.78% - 17.78% for BG. Foaming stability was found to be in the range of 7.22%-12.78%, 6.67%-13.89% for PG and BG (Figure 12). Results also suggested foaming capacity and foaming stability of both the samples increased with increased in concentration of gelatin. Similar results were observed by Dara et al., (2020) where foaming capacity and foaming stability of tuna skin gelatin depicted a direct relationship with gelatin concentration. Foam formation is a result of unfolding and rearrangement of smaller molecular weight peptide chain at air-liquid interface. Hydrophobicity of peptide and foaming capacity are reported to have a positive correlation

(Tkaczewska et al., 2018). Weak foaming properties of gelatin can also be due to degradation due to extraction process leading to generation of higher amount of low molecular weight peptide. These low molecular weight peptide lack in the ability of fostering film at air-water interface (Jin et al., 2019).

Fig. 12. Percentage of (a) foaming capacity and (b) foam stability of Pink Perch by-products gelatin (PG) and bovine gelatin (BG). Values presented as average of three measurements with SD.

3.7 Emulsion Activity Index and Emulsion Stability Index

Emulsion systems are generated by adsorption of peptides on the surface of oil droplet during homogenization, followed by formation of protective layer to prevent coalescing of the oil droplet (Duan et al., 2018). Effect of gelatin concentration on Emulsion Activity Index (EAI) and Emulsion Stability Index (ESI) was examined in this study. The EAI values of PG diminished with increase in gelatin concentration. However, a positive correlation was observed between ESI and gelatin concentration. Similar patterns of EAI and ESI were observed with BG with variation gelatin concentration. The PG showed highest EAI ($13.2 \pm 0.09 \text{ m}^2 \text{ g}^{-1}$) at 0.1% (w/v) of gelatin concentration and highest ESI ($10.0 \pm 0.0 \text{ Min}$) at 1% (w/v) of gelatin concentration. These

values were considerably lower than that of BG which depicted highest EAI of $38.4 \pm 0.25 \text{ m}^2\text{g}^{-1}$ and ESI of $8.61 \pm 0.04 \text{ Min}$ (Figure 13). Emulsion properties of gelatin can be attributed to its amphoteric nature (Koli et al., 2012). Emulsion properties of a gelatin majorly is determined by gelatin composition, intrinsic properties, and conformation of gelatin (Dara et al., 2020). Decrease in EAI at higher gelatin concentration can be due to higher interaction of gelatin with each other due to higher hydrophilicity rendering lesser gelatin molecules for distribution at air-water interface (Jin et al., 2019). Amount of higher molecular weight peptide and content of hydrophobic amino acid residues positively influence emulsion stability. Also, higher extent of intact α and β peptide chain residue results in greater interaction with oil in system leading to improved stability of emulsion (Duan et al., 2018).

Fig. 13. (a) Emulsification activity index and (b) emulsification stability index of Pink Perch by-products gelatin (PG) and bovine gelatin (BG). Values presented as average of three measurements with SD.

3.8 Color

Color is an important parameter in determining acceptability of food by aesthetically appealing to consumer and acts as a quality indicator of physio-chemical changes in food. The color of PG

was expressed in terms of L* (Lightness), a* (redness/greenness), and b* (yellowness/ blueness) as 86.0±0.3, 0.1±0.0, 8.6±0.0 respectively as compared to L* value of 71.1±2, a* value of 5.4±0.2, and b* value of 17.7±0.3 of BG (table 6). Higher L* and lower a*, b* value of PG is suggested proximity to white color. Whereas lower L* value coupled with higher a* and b* value indicated darker color of BG. Darker color in gelatin can be attributed to presence of may also indicate incomplete removal of mucosubstance removal during extraction process (Tkaczewska et al., 2018). Lighter color of gelatin is desirable attribute as it will be easier to incorporate lighter color compound in food without altering its aesthetic appeal.

Table 6

Color parameter of Pink Perch by-products gelatin (PG) and bovine gelatin (BG)

	PG	BG
L*	86.0±0.3	71.1±2.1
a*	0.1±0.0	5.4±0.2
b*	8.6±0.0	17.7±0.3
C*	8.6±0.0	18.5±0.2
h*	89.0±0.3	73.2±0.8

Values are means ± standard deviation (n=3)

3.9 Amino acid composition

Amino acid composition of PG and BG are illustrated in table 7. A higher concentration of imino acids of 18.1% (proline + hydroxyproline) was observed in PG in comparison to 4.79% imino acid in BG. Proline and hydroxyproline play a vital role in providing stability to triple helical structure of gelatin (Karim et al., 2009) and improve visco-elastic properties subsequently leading to formation of strong gel structure (Gomez-Guillen et al., 2002). Hydroxyl group present in imino acid especially hydroxyproline interact with water molecules and form hydrogen bond with carboxyl group. Imino acid content in fish are greatly dependent on environmental temperature of fish habitat since at higher temperature greater amount of collagen molecules are present to provide thermal stability to fishes. Also, charged amino acid (arginine + glutamic acid + aspartic acid + lysine) constituted approximately 43.7%. Charged amino acids contribute in inter and intramolecular interaction occurring during formation of triple helix

553 structure in gelatin. PG gelatin composed of 46.4% essential amino acids in comparison to
 554 39.32% of essential amino acid content of BG.

555 Table 7:

556 Amino Acid Profile of Pink Perch by-products gelatin (PG) and bovine gelatin (BG)

Amino Acid	PG	BG
Taurine	nd	1.27
Methionine sulfoxide	nd	1.44
Hydroxyproline	7.4	0.23
Aspartic acid + Asparagine	0.1	7.37
Threonine	nd	4.54
Serine	0.2	6.32
Glutamic acid + Glutamine	0.9	12.70
Proline	10.7	4.54
Lysine	12.7	6.57
Alanine	10.0	10.75
Valine	2.2	5.78
Methionine	1.6	2.55
Isoleucine	1.5	4.39
Leucine	3.6	8.95
Tyrosine	0.9	2.92
Phenylalanine	2.9	5.80
Histidine	2.0	1.79
Hydroxylysine	nd	0.06
Glycine	32.6	5.52
Ammonia	3.1	2.20
Arginine	10.1	4.19
SUM (AA* only)	100.0	100.00
TEAA**	46.40	39.32

557 *AA= Amino Acid ** TEAA = Total Essential Amino Acid

3.10 FT-IR Spectra

FTIR spectra has been used to monitor the specific functional groups and secondary structural conformation of gelatin sample. Several peaks were observed in the region of 4000-500 cm^{-1} which majorly depicts amide A, B, I, II and III (Figure 14, table 8).

Both gelatin samples exhibited amide A near 3400 cm^{-1} indicating N-H stretching coupled with H-bonding and amide B representing vibration modes of asymmetric stretch of $=\text{C}-\text{H}$ and $-\text{NH}_3$ at 2900 cm^{-1} . Peak for Amide I was observed at 1600 cm^{-1} are caused by $\text{C}=\text{O}$ stretching coupled with $\text{C}-\text{N}$ stretch and CCN deformation. For amide II, two peaks were observed at 1550 cm^{-1} and 1400 cm^{-1} , which were attributed to a combination of N-H deformation vibration and the $\text{C}-\text{N}$ stretching vibration. Another peak of Amide III was observed at 1200-1100 cm^{-1} which was corresponding to vibrations from CH_2 group glycine backbone and proline sidechain. Presence of amide III peak depicted disorganized gelatin structure. This phenomenon is associated with interruption in interchain reactions caused by extraction parameters leading to loss of triple helix structure. The peaks at amide I, II and III are commonly associated with the secondary protein structure present in the skin gelatin (Sockalingam et al., 2016). The wavenumber and amplitude of the major peaks in amide regions were found to be similar, implying that the secondary structure and functional groups of PG and BG are similar.

Table 8:

FTIR peak locations for Pink Perch by-products gelatin (PG) and bovine gelatin (BG)

Region	PG	BG
Amide A	3418	3425
Amide B	2925	2919
Amide I	1640	1638
Amide II	1558, 1415	1561, 1415
Amide III	1236	1102

Fig 14: FTIR Spectra of Pink Perch by-products gelatin (PG)(Red) and Bovine gelatin (BG) (Black)

3.11 SEM Analysis

The microstructures of PG and BG are illustrated in Fig. 15. PG has spherical and dense structure. Whereas BG showed thinner strands, such a gel network had the less resistance to the force applied, leading to a lower gel strength. In general, the conformation and association of protein molecules in gel matrices directly contribute to gel strength of gelatin (Benjakul et al., 2009). The thinner strand network had less inter-connected protein chains than the denser counterpart, resulting in weaker gel strength.

Fig 15: Microstructures of 6.67% gelatin gels from Pink Perch by-products gelatin (a, b) and bovine gelatin (c, d) at 1000 $\times$ and 3000 $\times$ magnification respectively.

4 Conclusion

In this study, gelatin extraction from industrial by-products of Pink Perch was conducted using single step extraction method with acetic acid. This process was optimized using Response Surface Methodology at different pH, temperature, and time to maximize yield and L-hydroxyproline content. The yield of gelatin obtained at optimized condition was 16.2% with high gel strength of 793g. Moisture and ash content in the gelatin obtained at optimized condition was within the FSSAI standards of edible gelatin. Use of organic acid makes optimized

method cost effective and environmentally sustainable. Further, Pink Perch by-products gelatin obtained by this method exhibited fine emulsifying activity, foam expansion, water- and oil absorption capacity suggesting its potential application for development of various food systems. The physicochemical and rheological properties of the Pink Perch by-products gelatin were comparable to the control bovine gelatin. Thus, providing a sustainable alternative of mammalian gelatin along with added benefits of reduced risk of pathogenic vectors such as prions and greater acceptability from Kosher and Halal consumers.

5 Conflict of Interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

6 Acknowledgement

NK and Khushboo would like to acknowledge the help extended by Dr. Shalini Rudra, Principal Scientist, IARI, for analysis of gelatin. Authors are also indebted to Department of Biotechnology, Government of India for providing the grant no. BT/IN/INNO-INDIGO/12/NK/2017-18 & 21/2/2018 to Dr. Nutan Kaushik for conducting the research.

7 Contribution of Author's

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

8 Data Availability

The datasets generated during and/or analysed during the current study is present in manuscript and has no further associated data.

9 References

Abdelhedi, O., Nasri, R., Mora, L., Toldrá, F., Nasri, M., & Jridi, M. (2017). Collagenous proteins from black-barred halfbeak skin as a source of gelatin and bioactive peptides. Food Hydrocolloids, 70, 123-133.

625 Benjakul, S., Oungbho, K., Visessanguan, W., Thiansilakul, Y., & Roytrakul, S. (2009).
626 Characteristics of gelatin from the skins of bigeye snapper, *Priacanthus tayenus* and *Priacanthus*
627 *macracanthus*. *Food Chemistry*, 116(2), 445-451.

628 Bergman, I., & Loxley, R. (1963). Two improved and simplified methods for the
629 spectrophotometric determination of hydroxyproline. *Analytical chemistry*, 35(12), 1961-1965.

630 Boran, G., Mulvaney, S. J., & Regenstein, J. M. (2010). Rheological properties of gelatin from
631 silver carp skin compared to commercially available gelatins from different sources. *Journal of*
632 *food science*, 75(8), E565-E571.

633 Cho, S. M., Gu, Y. S., & Kim, S. B. (2005). Extracting optimization and physical properties of
634 yellowfin tuna (*Thunnus albacares*) skin gelatin compared to mammalian gelatins. *Food*
635 *Hydrocolloids*, 19(2), 221-229.

636 Dara, P. K., Raghavankutty, M., Sebastian, N., Chatterjee, N. S., Mathew, S., Ravishankar, C.
637 N., & Anandan, R. (2020). Rheological, Physico-chemical, and Surface-Active Properties of
638 Gelatin Extracted from Bigeye Tuna (*Thunnus obesus*) Skin Waste. *Journal of Aquatic Food*
639 *Product Technology*, 29(5), 428-444.

640 Duan, R., Zhang, J., Liu, L., Cui, W., & Regenstein, J. M. (2018). The functional properties and
641 application of gelatin derived from the skin of channel catfish (*Ictalurus punctatus*). *Food*
642 *chemistry*, 239, 464-469

643 Elavarasan, K., Kumar, A., Uchoi, D., Tejpal, C. S., Ninan, G., & Zynudheen, A. A. (2017).
644 Extraction and characterization of gelatin from the head waste of tiger tooth croaker (*Otolithes*
645 *ruber*). *Waste and biomass valorization*, 8(3), 851-858.

646 FAO. 2020. The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome.

647 Gómez-Guillén, M. C., Turnay, J., Fernández-Díaz, M. D., Ulmo, N., Lizarbe, M. A., &
648 Montero, P. (2002). Structural and physical properties of gelatin extracted from different marine
649 species: a comparative study. *Food Hydrocolloids*, 16(1), 25-34.

650 Hue, C. T., Hang, N. T. M., & Razumovskaya, R. G. (2017). Physicochemical Characterization
651 of Gelatin Extracted from European Perch (*Perca fluviatilis*) and Volga Pikeperch (*Sander*
652 *volgensis*) Skins. *Turkish journal of fisheries and aquatic sciences*, 17(6), 1117-1125.

653 Jin, W. G., Pei, J., Du, Y. N., Pan, J., Gao, R., Chen, D. J., & Zhu, B. W. (2019).
654 Characterization and Functional Properties of Gelatin Extracted from Chinese Giant Salamander
655 (*Andrias Davidianus*) Skin. *Journal of Aquatic Food Product Technology*, 28(8), 861-876.

656 Karim, A. A., & Bhat, R. (2009). Fish gelatin: properties, challenges, and prospects as an
657 alternative to mammalian gelatins. *Food hydrocolloids*, 23(3), 563-576.

658 Koli, J. M., Basu, S., Nayak, B. B., Patange, S. B., Pagarkar, A. U., & Gudipati, V. (2012).
659 Functional characteristics of gelatin extracted from skin and bone of Tiger-toothed croaker
660 (*Otolithes ruber*) and Pink perch (*Nemipterus japonicus*). *Food and bioproducts processing*,
661 90(3), 555-562.

662 Krul, E. S. (2019). Calculation of nitrogen-to-protein conversion factors: A review with a focus
663 on soy protein. *Journal of the American Oil Chemists' Society*, 96(4), 339-364.

664 Kumar, D. P., Chandra, M. V., Elavarasan, K., & Shamasundar, B. A. (2017). Structural
665 properties of gelatin extracted from croaker fish (*Johnius* sp) skin waste. *International journal of*
666 *food properties*, 20(sup3), S2612-S2625.

667 Mafazah, E. M., Pranoto, Y., & Rohman, A. (2018, March). Extracting of yellowfin tuna
668 (*Thunnus albacares*) fish skin gelatin as influenced by alkaline concentration and extraction
669 times. In *IOP Conference Series: Earth and Environmental Science* (Vol. 139, No. 1, p. 012047).
670 IOP Publishing.

671 Martins, M. E. O., Sousa, J. R., Claudino, R. L., Lino, S. C. O., Vale, D. A. D., Silva, A. L. C., &
672 De Souza, B. W. (2018). Thermal and chemical properties of gelatin from tilapia (*Oreochromis*
673 *niloticus*) scale. *Journal of Aquatic Food Product Technology*, 27(10), 1120-1133.

674 Mi, H., Wang, C., Chen, J., Xu, Y., Li, X., Li, J., ... & Lao, M. (2019). Characteristic and
675 functional properties of gelatin from the bones of alaska pollock (*Theragra chalcogramma*) and
676 yellowfin sole (*Limanda aspera*) with papain-aided process. *Journal of Aquatic Food Product*
677 *Technology*, 28(3), 287-297.

678 Mirzapour-Kouhdasht, A., Moosavi-Nasab, M., Krishnaswamy, K., & Khalesi, M. (2020).
679 Optimization of gelatin production from Barred mackerel by-productss: Characterization and
680 hydrolysis using native and bovine proteases. *Food Hydrocolloids*, 108, 105970.

681 Osnes, K. K., & Mohr, V. (1985). On the purification and characterization of three anionic,
682 serine-type peptide hydrolases from Antarctic krill, *Euphausia superba*. *Comparative*
683 *Biochemistry and Physiology Part B: Comparative Biochemistry*, 82(4), 607-619.

684 Singh, R. K., & Balange, A. K. (2005). Characteristics of pink perch (*Nemipterus japonicus*)
685 surimi at frozen temperature. *Journal of food processing and preservation*, 29(1), 75-83.

686 Sockalingam, K., Nelson, H., Idris, M. I., & Abdullah, H. Z. (2016). Effects of pre-treatment
687 durations on properties of black tilapia (*Oreochromis Mossambicus*) skin gelatin. In *Materials*
688 *Science Forum* (Vol. 840, pp. 146-150). Trans Tech Publications Ltd.

689 Tan, C. C., Karim, A. A., Uthumporn, U., & Ghazali, F. C. (2019). Effect of extraction
690 temperature on the physicochemical properties of gelatine from the skin of black tilapia
691 (*Oreochromis mossambicus*). *Journal of Physical Science*, 30, 1-21.

692 Tkaczewska, J., Morawska, M., Kulawik, P., & Zając, M. (2018). Characterization of carp
693 (*Cyprinus carpio*) skin gelatin extracted using different pretreatments method. *Food*
694 *Hydrocolloids*, 81, 169-179.

695 Yang, H., Wang, Y., Jiang, M., Oh, J., Herring, J.L., & Zhou, P. (2007). 2-step optimization of
696 the extraction and subsequent physical properties of channel catfish (*Ictalurus punctatus*) skin
697 gelatin. *Journal of food science*, 72 4, C188-95.

698 Zhang, T., Sun, R., Ding, M., Tao, L., Liu, L., Tao, N., & Zhong, J. (2020). Effect of extraction
699 methods on the structural characteristics, functional properties, and emulsion stabilization ability
700 of Tilapia skin gelatins. *Food Chemistry*, 328, 127114.