

Novel method for gelatin extraction of various local fish using High Pressure Processing (HPP)

^{1,2,3*}Jaswir I., ¹Yusof N., ¹Jamal P. and ¹Jami M. S.

¹Department Biotechnology Engineering, Kulliyah of Engineering, International Islamic University Malaysia, 68100 Kuala Lumpur, Malaysia.

²International Institute for Halal Research and Training (INHART), International Islamic University Malaysia, 68100 Kuala Lumpur, Malaysia

³Marine Natural Product Research Centre, Surya University, Tangerang, Indonesia

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Abstract

Gelatin from fish skin is known to be an alternative source for mammalian gelatin. However, it has weaker properties compared to bovine and porcine gelatin, which limits its use in the industry. The conventional method for fish gelatin extraction requires long production time and could cause serious water pollution and chemical treatments are often being used to enhance the yield of fish gelatin and its properties but it may affect the amino acid content of the gelatin. In this regard, High-Pressure Processing (HPP) is a novel method suggested for fish gelatin extraction. The HPP method is classified as green technology as it requires low electricity throughout the process. This study will discuss the impact of HPP the technique gelatin extracted from fish skin. Skins from four types of fish, namely red tilapia (*Oreochromis niloticus*), black tilapia (*Oreochromis mossambicus*), grouper (*Epinephelus areolatus*) and threadfin bream (*Nemipterus tambuloides*), were used. High pressure was applied at either pre-treatment in citric acid solution or during thermal extraction; and the pressure was maintained at 250 MPa with pressure holding time of 10 minutes and 18 hours of water extraction. Gelatin extract from traditional acid-base method was prepared as a standard for comparison. The study found that there was an increment in the yield of gelatin and the concentration of gelatin extract, and the pre-treatment time was also reduced.

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Keywords

Fish gelatin
Green technology
High pressure extraction
Protein hydrolysis
FTIR

Introduction

Gelatin is formed from collagen through partial hydrolysis process and normally found in bone, tendon and skin of mammals (Fratzl, 2008). The collagen structure consists of three polypeptide chains (Gly-X-Y) with repeated sequences. X and Y mainly occupied by proline and hydroxyproline residues (Hulmus, 2008). Each chain has a left-handed folded poly-L-proline conformation, and three helices are folded together in a right-handed coil-coil. The triplets' structure is stabilized by one or two inter-chain hydrogen bonds. In addition, the exposure of proline and hydroxyproline residues to the solvent will cause the triple The traditional acid-base extraction process of gelatin from fish skin consists of three parts, which are pre-treatment, extraction and purification. In the pre-treatment process, acid and alkali were used to remove non-collagenous materials and for the swelling process. Partial cleavage of crosslink occurred when samples are treated with mild acid, resulted in the structural changes that enable the production of water soluble

collagen (gelatin) (Schrieber and Gareis, 2007).

The critical need for halal gelatin leads to numerous research on alternative gelatin source. Fish skin is known to have high gelatin content due to the fact that fish is the main source of protein (Elgadir *et al.*, 2013). Besides that, the use of fish gelatin can solve religious issues (Islam, Jew, vegetarian, Hinduism) and concerns on health problems (mad cow and BSA) (Arnesen and Gildberg, 2007). However, the properties of gelatin extracted from fish skins are not as excellent as those from mammals (Benjakul *et al.*, 2012). Therefore, the modification of gelatin structure is needed to improve the protein molecules. Chemical modifications are common but they could generate chemical wastes and may affect the amino acid contents of the gelatin (Liqing *et al.*, 2012). Hence, some researchers have suggested the physical modification method known as 'High-Pressure Processing' (HPP) (Gudipati and Kannuchami, 2014).

High Pressure Processing (HPP), also known as Ultra-High Pressure, UHP and High Hydrostatic Pressure, HHP, is a non-thermal preservation

*Corresponding author.
Email: irwandi@iium.edu.my

technique to inactivate the microorganisms in food, without altering their freshness and the nutritional value (Barba *et al.*, 2012). Even though the main application of HPP system is for food preservation, recently HPP was introduced as an alternative method for gelatin extraction (Rastogi *et al.*, 2007). Imposing high pressure on the foodstuff can persuade the modifications of food macromolecules. Studies have shown that there were changes in the meat texture and tenderness throughout pressurization, as a results of the modification of collagen properties, such as collagen contents, solubility and thermal stability (McArdle *et al.*, 2011; Niu *et al.*, 2013). In this light, no significant changes in collagen behaviour were testified when pressure (up to 500MPa) were applied, and it appears to strengthen the protein structure at a certain pressure (Gómez-Guillén *et al.*, 2005). In addition, the presence of high pressure could induce protein denaturation (Basu, 2016) and extreme mechanical loads distress the balance of the non-covalent bond interactions that stabilize the native configurations of many proteins, which later make it easier for gelatin to be extracted (Chen *et al.*, 2014). Moreover, the presence of high pressure was reported to assist the swelling process by allowing more acid to penetrate into the interiors of proteins with increasing pressure, thus, increasing the yield of the gelatin extracts (Sarupria *et al.*, 2010). Other advantages of HPP in the extraction process are first, it can reduce more than 50% of extraction time (Zhang *et al.*, 2011), seconds avoid heat degradation since no thermal process involve in the process and finally high pressure acts equally on a food mass regardless of its size, shape and composition (Prasad *et al.*, 2012).

In this study, the effects of high pressure on the gel formation and the percentage yield of gelatin extracts from fish skin have been examined. Skin from four different types of fish were used in this project; red tilapia, black tilapia, grouper and threadfin fish. Fish gelatin was extracted using the conditional method stand as standard.

Materials and methods

Materials preparation

The fishes (red tilapia (*Oreochromis niloticus*), black tilapia (*Oreochromis mossambicus*), grouper (*Epinephelus areolatus*) and threadfin bream (*Nemipterus tambuloides*) were bought from a supermarket in Gombak and their flesh and bone were removed while their skins were washed, cleaned and cut into squares (1 cm x 1 cm) before being stored in -20°C until further use. Three solutions, 0.2% NaOH,

0.2% acetic acid (C₂H₄O₂) and 1.0% citric acid (C₆H₈O₇) were prepared and kept in 4°C for at least overnight. Analytical grade chemicals used were in the whole process.

Gelatin extraction: Acid-base extraction (S1)

The fish skins were thawed and cleaned and the gelatin extraction procedure was carried out according to (Grossman and Bergman, 1992) with slight modification. During the pre-treatment process, the skins were soaked in NaOH, acetic acid and citric acid, respectively. Each soaking period lasted for 40 minutes at 4°C. The skins were washed thoroughly using distilled water after every soaking. The ratio for the skin and all solution was 1:14. Later, treated fish skin was extracted in distilled water for 18 hours at 45°C. The supernatant was kept for further analysis while precipitated components were discarded.

Gelatin extraction: HPP in pre-treatment (S2)

The procedure for gelatin extraction assisted by HPP in the pre-treatment process was performed according to (Gómez-Guillén *et al.*, 2005) with several adjustments. Each fish skin was soaked in NaOH and acetic acid, similar to the previous method. For the swelling procedure, the skins and citric acid were sealed in a polyethylene bag. The bag was placed inside the pressure chamber and the lid was closed. HPP was run at the pressure 250 MPa for 10 min, followed by thermal extraction in distilled water (as conventional method).

Gelatin extraction: HPP in extraction (S3)

The skins were soaked in NaOH, acetic acid and citric acid as mention in conventional methods. Later, samples of the fish skins are put into distilled water in a sealed polyethylene bag, which later being placed into the pressure chamber and HPP method was performed for 10 min at 250 MPa. Supernatant was kept for further analysis while precipitate being discarded. Summary of S1, S2 and S3 process illustrated in Figure 1.

Gel observation

Gelatin extract was kept at 4°C overnight. This will preserve the gelling formation of the gelatin extract (Krug, 2012). Gel formations for each sample were recorded.

Protein concentration (Biuret test)

The biuret test was done according to Gornall *et al.* (1949). UV-Vis Spectrophotometer (Brand: Biochrom, Model: LIBRA S12) were used to obtain the absorbance reading for each concentration at 540

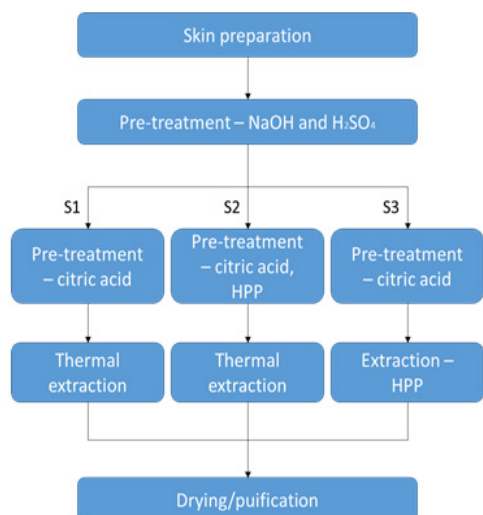


Figure 1. Summary of different extraction process

nm wavelength.

Fourier Transform Infrared Spectroscopy (FTIR) analysis

The functional groups and secondary structure of gelatin extract were done by using The Thermo Scientific Nicolet iS50 FT-IR spectrometer.

Results and discussion

Sample preparation

Four types of fish (red tilapia (RT), black tilapia (BT), grouper (G) and threadfin bream (TB)) were used in this research. Skins from the fishes were kept in -20°C freezer overnight to strengthen the gel strength of the gelatin in the skin (Karim and Bhat, 2009), while chemical solutions (sodium hydroxide, acetic acid and citric acid) were stored at 4°C to avoid any gelatin loss during the pre-treatment (Ademola, 2010).

Precautions while handling HPP machine

HPP is a sensitive machine, hence there are certain precautions that need to be taken into consideration while handling the HPP procedure to avoid damages to the process. For instance, maintaining the water level is important as water is used as a pressure medium. Insufficient amount of water will damage the HPP system. In addition, it is critical to remove all bubbles from the polyethylene plastic bag before it is sealed. High pressure exerted on the bubbles will make the bag burst out during HPP procedure. Particles from the bag will come out and disrupt the machine, thus, affecting the HPP process. Through the observation, removing bubbles from citric acid solution is easier compared to removing them from distilled water. Moreover, attention also should be given to the quantity of the sample while running the

Table 1. Physical appearance of gelatin extract

Fish types	Process	pH	Colour of extract solution	% of skin weight loss
Red tilapia	S1	4.24 ± 0.070	Colourless	57.79 ± 0.17
	S2	4.36 ± 0.189	Colourless	60.62 ± 0.361
	S3	3.75 ± 0.025	Brownish	+112 ± 0.424
Black tilapia	S1	4.63 ± 0.100	Yellowish	57.84 ± 3.677
	S2	4.43 ± 0.132	Yellowish	59.86 ± 0.481
	S3	4.06 ± 0.087	Light yellow	+98.2 ± 2.546
Grouper	S1	4.79 ± 0.167	Colourless	49.4 ± 0.297
	S2	4.68 ± 0.070	Colourless	52.58 ± 0.870
	S3	4.34 ± 0.629	Colourless	+60 ± 0.001
Threadfin bream	S1	4.72 ± 0.010	Brownish	59.5 ± 1.039
	S2	4.7 ± 0.064	Brownish	65.12 ± 0.792
	S3	4.42 ± 0.112	Brownish	+102.2 ± 1.061

HPP machine. Even though the vessel can hold up to 150 ml of samples, it is advisable not to fully occupy the tank so the pressure medium (water) could enter the vessel and execute the high pressure process.

Physical appearance

Table 1 presents the physical observation of gelatin extract obtained from the fish skins in different extraction methods. The pH results for gelatin from all fishes were acceptable as they were within the standard of edible gelatin, which are between 3.5 – 5.5 for Type A gelatin (GMIA, 2012). The pH number from gelatin extracted through high pressure assistance (S2) was not significantly difference from the control (S1). This is showing that pressurization during pre-treatment process does not affect the pH of the gelatin extract. Gelatin from S3 procedure obtained lower pH compared to S1 and S2. Until recently there are no explanation on the relationship between the pH of gelatin and the method used for its extraction (Park et al., 2013).

Colourless liquid appeared on the gelatin solution extracted from red tilapia and grouper skin while black tilapia and threadfin bream produced a brownish extract solution (Table 1). Hence, different fish species, fish origin and extraction method lead to various pH and colour of the gelatin extract solutions (Ratnasari et al., 2013).

The weight of each skin was taken before and after extraction. From the Table 1, final skin weight from the S2 course was lower compared to the standard (S1) method for all types of fish. For the red tilapia fish, the percentage of skin weight loss in S2

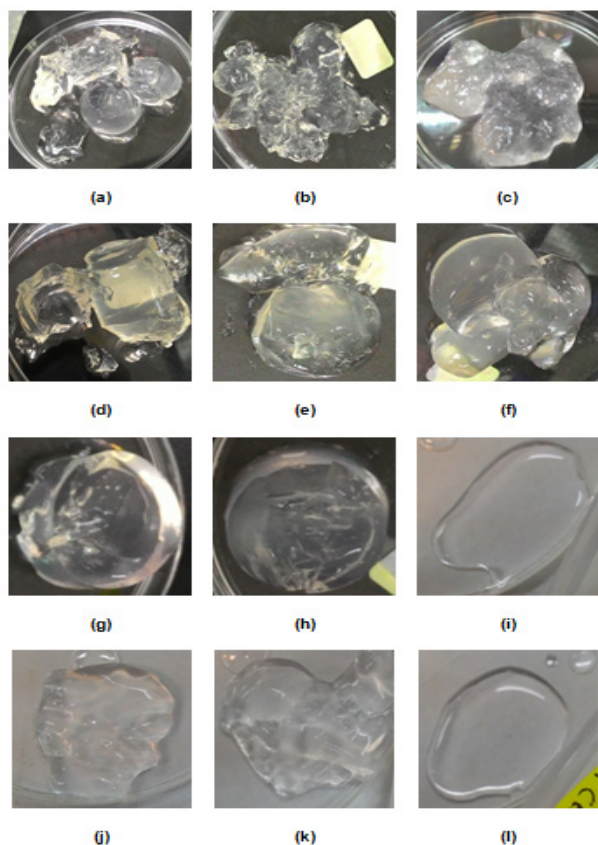


Figure 2. Gel formation of gelatin extracted using different methods. (a) Red tilapia: Conventional methods (RTS1); (b) Red tilapia: HPP pre-treatment (RTS2); (c) Red tilapia: HPP extraction (RTS3); (d) Black tilapia: Conventional methods (BTS1); (e) Black tilapia: HPP pre-treatment (BTS2); (f) Black tilapia: HPP extraction (BTS3); (g) Grouper: Conventional methods (GS1); (h) Grouper: HPP pre-treatment (GS2); (i) Grouper: HPP extraction (GS3); (j) Threadfin bream: Conventional methods (TRS1); (k) Threadfin bream: HPP pre-treatment (TRS2); (l) Threadfin bream: HPP extraction (TRS3).

process was 60.62% compared to 57.79% for the S1 method. In addition, 52.58% of grouper skin weight loss was recorded for the S2 course compared to 49.4% in S1. Threadfin Bream recorded the highest difference in the percentage of skin weight loss in S2 compared to standard, which were 65.12% and 59.5%, respectively. During S2, the presence of high pressure increased the swollenness of the fish skin during pre-treatment and permitting more water to infiltrate into protein structure, which allowing more gelatin to be extracted during thermal hydrolysis (Liqing *et al.*, 2014). Besides, the pressurization during pre-treatment destabilized the non-covalent bond interactions and during thermal extraction, the three polypeptide chains could easily break into single chain and increase the amount of gelatin extracts (Zhang *et al.*, 2016). On the other hand, final skin weight for S3 was doubled after the extraction process (Table 1). This is showing that a lot of gelatin

still has not been extracted after 10 minutes water extraction in the S3 process. More time is needed to extract the gelatin from the skin using an HPP method during protein hydrolysis.

Gel formation of gelatin

Extract solution of gelatin was kept at a matured temperature (4°C) overnight. Figure 2 shows the result of gel formation for all samples. Twelve samples of fish gelatin were produced from four types of fish skin, in three different processes. Gelatin extracted from the S1 method has been used as a standard. The gels from gelatin assisted by HPP in pre-treatment for red tilapia, black tilapia and grouper appear to have similar gel formation with the standard (S1) while gelatin from S3 process produced fragile gel/no gels. The gel formation in the gelatin extracts from black tilapia skin had the same observation as red tilapia. Meanwhile, grouper is known to have high gel strength (Irwandi *et al.*, 2009) and gels produced from S1 and S2 methods were solid, meanwhile no gels appeared at the S3 sample after being refrigerated. For the gelatin from the threadfin bream skin, S2 produced the gelatin slightly harder gel at 4°C compared to S1 process, and no gel at all for S3 sample.

The results from Figure 1 show that all gelatin from S3 process had failed to produce gels at lower temperature (4°C), indicating that all have very low melting points (Jones, 2004). Furthermore, even though the processing time were decreased from 18 hours to only 10 minutes during water extraction, it has no commercial value. Gómez-Guillén *et al.* (2005) suggested that the increasing the extraction time will improve the results and the amino acid content present in the gelatin helps in stabilizing the conformation of the gel formulation. In this regard, fish gelatin has lower proline and hydroxyproline concentrations compare to mammalian gelatin, which cause them to denature at low temperature (Mariod and Adam, 2013). The results show that the presence of high pressure (250MPa) does not degrade the gelatin protein. In fact, high pressure in pre-treatment method helps in enhancing the protein structure (Chang *et al.*, 2013).

Concentration of gelatin

Biuret test is a simple, rapid and precise qualitative test for protein determination in gelatin. Colour response on Biuret test depends on the molecular weight distribution and amino acid composition. The correlation of absorbance (y) and protein concentration (x) obtained from the standard curve was $y = 0.0553x$ ($R^2 = 0.9993$). As observed

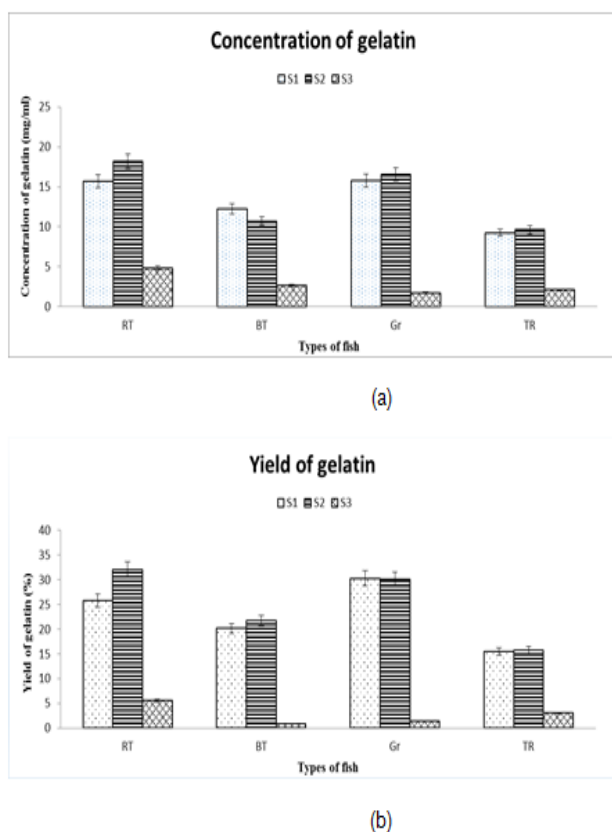


Figure 3. Results for (a) concentration and (b) percentage yield of gelatin for various types of fish

from Figure 3 (a), the concentration of gelatin from S2 process was higher compared to the standard (S1) for all types of fish and the red tilapia in S2 process exhibited the increment of the gelatin concentration more than 2% (18.21%) compared to standard (15.7%). Comparably, the protein concentration of gelatin from grouper and threadfin bream had also increased after high pressure treatment, from 15.78% to 16.67% and 9.25% to 9.61%, respectively. These findings proved that the use of high pressure is able to increase the concentration of the gelatin, indicating the improvement of the gelatin's properties. The pressurization hardly destroyed the amino acid composition in the gelatin but, it inherently affected the composition of amino acid and distribution of molecular weight of the gelatin (Rendueles *et al.*, 2011). The gelatin arrangement was modified slightly during HPP process, thus enhancing the concentration of the gelatin protein extracts and the concentrations of gelatin extracted from all types of fish during the S3 procedure were lower compared to the standard. This illustrates the ineffectiveness of this method and the need for more studies.

Yield of gelatin

The gelatin yield measurement was done by dividing the dry weight of gelatin extract to their initial skin weight. Results for the gelatin yield are

illustrated in Figure 3 (b). The S2 process produced higher yield compared to the S1 process for red tilapia and black tilapia fish skin, while grouper and threadfin bream produced similar yields for both processes. In this light, the increment of gelatin yield from red tilapia skin (from 258 mg/g S1 process to 321 mg/g S2 process) and 217.5mg/g from 201.5 mg/g for black tilapia skin was caused by the pressurization, high-pressure-induced protein denaturation by destabilizing the inter and intra molecular bond, which will increase the yield of the gelatin during thermal extraction (Liqing *et al.*, 2012). Besides, the presence of higher-pressure during pre-treatment allows more acid to penetrate into the skin structure. According to the mass transfer theory, the rate of mass transfer is equal to the pressure/resistance, hence higher pressure will increase the amount of solvent infiltrate into the cell membrane, and thus more acid could permeate the cell membrane. Under the process of HPP extraction, the differential pressure between the cell interior and the exterior of cell membranes is large that it will lead to rapid permeation (Shouqin *et al.*, 2005). For grouper and threadfin bream, there are no changes in the yield, which shows that the acid/HPP treatment done in this study does not have significant impact. Hence, changing the procedure or acid used might be the best solution in understanding the effect of HPP on gelatin yield. Besides that, even though the pressure does not have any significant influence on the yield, it might affect the other physical properties. A study done by Chang *et al.* (2013) found that the mechanical strength of the collagen increased even though the yield of collagen from skin treated by HPP is similar with the traditional acid/base extraction method. Thus, more study on gelatin properties are required to study the impact of HPP on gelatin extraction. Consequently, the yield from S3 process show poorer results for all samples. This is similar to the research done by Gómez-Guillén *et al.* (2005).

Fourier Transforms Infrared Spectroscopy (FTIR)

Fourier Transform Infrared (FTIR) spectroscopy is a method to determine the functional groups and the secondary structure of gelatin (Kaewruang *et al.*, 2013; Nikoo *et al.*, 2014). Generally, gelatin protein consist of five amide bands, which are amide A, Amide B, Amide I, Amide II, Amide III, which resulted from NH stretching vibration, asymmetry stretching CH, CO stretching, NH bending and CH stretching, respectively (Cebi *et al.*, 2016). The spectra of gelatin show the major peaks in the amide region. To compare the absorption intensity between the spectra, the peak height of all amide bands were

Table 2. Comparison of Fourier transforms infrared spectroscopy (FTIR)

Fish	Process	Wavenumber (cm ⁻¹)				
		Amide A	Amide B	Amide I	Amide II	Amide III
Grouper	S1	3296.89	2936.36	1636.68	1532.47	1233.88
	S2	3295.73	2934.47	1635.92	1532.14	1234.23
Black tilapia	S1	3296.8	2934.76	1635.69	1531.76	1231.72
	S2	3296.18	2935.89	1633.03	1539.75	1234.66
Red tilapia	S1	3297	2935.56	1635.96	1529.29	1231.5
	S2	3296.54	2931.13	1633.49	1539.42	1235.64
Threadfin bream	S1	3295.79	2935.49	1635.88	1529.51	1232.58
	S2	3289.44	2925.84	1632.33	1540.58	1237.45

quantitatively measured and compared (Kuan *et al.*, 2016). From the results in Table 2, wavenumber of gelatin protein from fish skin that has underwent pressurization produced a peak similar to the gelatin extracted using traditional acid-base extraction. For example, amide I, II, III, A and B for gelatin extracted from threadfin bream fish skin using conventional method were 1635.88 cm⁻¹, 1529.51 cm⁻¹, 1232.58 cm⁻¹, 3295.73 cm⁻¹ and 2935.49 cm⁻¹, respectively, while 1632.33 cm⁻¹, 1540.58 cm⁻¹, 1237.45 cm⁻¹, 3289.44 cm⁻¹ and 2925.84 cm⁻¹ were recorded for gelatin with HPP treatment. These results verified that gelatin structure was preserved even after experiencing the high pressure treatment.

Conclusion

Findings from this study have shown that high pressure processing during the swelling procedure is beneficial to reduce the extraction time (from 40 min to 10 min), increase the yield and enhancing the quality of the gelatin obtained (concentration of protein extract). Gelatin extracted from red tilapia skin provides significance result compared to grouper and threadfin bream in HPP method. More studies are needed to optimize the production of gelatin extract assistance by the HPP.

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