Halal gelatin extraction from Patin fish bone (*Pangasius hypophthalmus*) by-product with ultrasound-assisted extraction

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**Abstract.** The most gelatin is derived from porcine skins and bones that accounted for 46% of total gelatin. This is an obstacle to the development of food products in a Muslim country like Indonesia. One of the prospective sources to be developed is fish bones that account for 10-20% of fish weight, with the highest cultivation being *Pangasius* (Patin fish). This study aims to extract the gelatin from fish bone by-product with ultrasound-assisted extraction using a combination of 3, 5, and 7 hours of extraction time. Based on the results, 5 hours extraction time became the best treatment that gives the highest yield, that is 5 ± 1.03% with a value of gel strength, viscosity, and pH respectively 147.74 ± 0.83 g Bloom, 14.63 ± 0.31 cP, and 6.76 ± 0.3. Analysis of functional group with Fourier transform infrared (FTIR) has given a typical uptake of gelatin with the appearance of the amide peak. The result of molecular weight analysis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) obtained gelatin molecular weight with range 120.08-155.82 KDa.

1. **Introduction**

Gelatin is a biopolymer produced by extraction and partial hydrolysis from collagen. It has wide applications in the food and non-food industry such as pharmaceutical, cosmetics, biomedical, and photography. In biomedical gelatin is used as artificial skin material [1], antihypertensive and antioxidant [2] and inhibitors of angiogenic diseases such as complications of diabetes, obesity, and arthritis [3]. In the pharmaceutical industry, gelatin is used as a wound dressing, implant, and drug delivery [4]. Meanwhile, in the milk processing industry, they used gelatin as a foam-forming agent in ice cream, improves texture, product stability, and avoids the occurrence of syneresis, which is the loss of elasticity in yogurt and soft cheese.

Gelatin worldwide production reached 326,000 tons, with gelatin extraction of porcine skins (46%), bovine hide (29.4%), pork and cattle bones (23.1%) and other sources around 1.5% [5]. Global industry analysis predicts world demand for gelatin in 2022, is expected to increase to 480,000 tons based on its application in food and pharmaceutical fields. Indonesia imports gelatin from several countries to meet domestic gelatin requirement. Data from [6] in 2013, stated that imports of gelatin reached 280,771 kg as much as 2,210,812 US Dollar.

The use of gelatin from porcine became a barrier to the development of food product in countries with a majority of the Muslim population, such as Indonesia. Indonesia is a country that is very concerned about the safety and halal of products consumed, as well as the use of commercial gelatin in various applications. One of halal gelatin source is from bovine. However, this source still has flaws,
such as the price is quite high, and the news is about mad cow disease or Bovine spongiform encephalopathy (BSE) [8]. Given the wide use of gelatin in various industrial fields, to overcome this problem, it is necessary to develop safe raw materials, guarantee halal sources, and also reduces the number of imports.

Gelatin can be obtained from sources that contain collagen, such as skin, scales, and bones [9]. The seafood processing industry usually produces an amount of by-product, such as skins, bones, and scales with a high amount of collagen. This source can be developed as raw material for extraction of gelatin, especially the bones which contributes 10-20% of fish weight. Fish bones can be an alternative to halal gelatin that is accepted by the public. At present, the utilization of catfish bone waste has not been done optimally. Patin fish is one of the freshwater fish that is widely cultivated because of the high demand for domestic and international markets [10]. Based on data from the Ministry of Maritime Affairs and Fisheries, national patin fish production increased every year as much as 437,111 tons in 2017 and production target in 2018 is 604,587 tons. Increased catfish cultivation correlates with an increase in the catfish industry processed into filet [11]. This will be in harmony with the amount of bone waste produced. Optimization of this waste can avoid environmental problems and increase the added value of the fish processing industry.

Previous studies related to gelatin extraction with percent of yield from fish bones have been carried out using tuna (8.37%), tilapia (13.27), and snapper (9.4%) [11-13], but the yield obtained is still classified as low yield. The yield value depends on the initial treatment, the process, and the extraction method performed [14]. The extraction method that is often used is maceration, so it is necessary to develop methods to increase yield and improve the physicochemical properties of gelatin. Sonication is an extraction method that utilizes ultrasonic waves. Based on the research of [15,16], the sonication method can accelerate the extraction time, increased yield, and produced gelatin with excellent physicochemical properties. Therefore, in this study, the extraction method that will be used is sonication with initial treatment in the form of acid immersion. In addition, optimization related parameters such as extraction time need to be done.

2. Materials and method

The samples used were frozen patin fish bones provided by Vessel Fresh fish Indomakmur Corps. The bones were boiled in water at 60-70 °C for 30 minutes and washed in tap water to remove impurities and cut into 2 cm segments after the removal of residual and then stored in 4 °C. The chemical used is HCl 37 % analytical grade.

2.1. Preparation and extraction of gelatin from patin fish

Fish bones demineralized with 5% HCl at a ratio of 1:7(w/v) for 48 hours. The bones were then neutralized by washing them under tap water until their pH reached 7 and then washed again with distilled water to remove any residuals of tap water. The fish bones were mixed with distilled water at a ratio of 1:8 (bone/water (w/v)). The gelatin was extracted with ultrasound water bath instrument (Cole-Parmer 8893) upon 700 w at 70°C for varied times 3 h, 5 h, and 7 h, named UB3, UB5, and UB7 respectively. The mixture was filtered by filter paper, and a gelatin solution was dried by vacuum evaporator. Then all dried fish bones gelatin samples were weighed and subjected to further analysis. Gelatin yield was calculated using the following equation below:

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\text{Yield (\%)} = \frac{\text{weight of dried gelatin (g)}}{\text{weight of dried bones (g)}} \times 100\%
\] (1)
2.2. Determination of gel strength
Gel strength was determined according to methods of [17] using a Texture analyzer (TA-xt2i texture analyzer: probe 0.5 R Delrin; speed 1 mm/s; distance 10 mm; trigger auto 5 g). The dried gelatin was dissolved in distilled water at 50 °C to obtain a final concentration of 6.67% (w/v). Gelatin solution was placed in 25 mL glass beaker, and then cooled in a refrigerator at 4 °C and matured for 16 h. Gel strength was expressed as maximum force (in g) when the plunger had penetrated 4 mm into gelatin gels. Each sample was tested for two times.

2.3. Determination of viscosity
Viscosity was determined according to standard methods of [17] using a final concentration of 6.67% (w/v) of gelatin solution. The gelatin viscosity was determined by using Viscometer TV-10 (the shear rate at 100 rpm and spindle Number 2) at 60 °C gelatin solution. The viscosity was expressed in centipoise (cP).

2.4. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)
SDS-PAGE was done according to [18], SDS-PAGE analysis for gelatin using 3% stacking gel and 15% separating gel. Separating gel was added as much as 5 mL, then added 1 mL of distilled water. The gel is incubated at room temperature for 30 minutes until it hardens then the distilled water is added. Stacking gel is added as much as 1 mL and incubating in the refrigerator for overnight. Gelatin samples as much as 2 mg were dissolved with 1 mL SDS 5%, then heated at 85 ºC for 1 hour then centrifuged for 5 minutes with a speed of 12400 G. After that it was taken as much as 20 µL and added a sample buffer with a ratio of 1:1 (v/v) then heated at 85 ºC for 10 minutes. Samples and markers were loaded into the well as much as 9 µL and 15 µL respectively, which were connected to electric current at 15 A and 100 V for 3 h. Then the gel was soaked in 25 mL of staining solution for about 2 hours and rinsed with water, then soaked with 50 mL of the destaining solution until the protein band was clearly visible. The resulting gel is analyzed for molecular weight using Photocap software.

2.4. Fourier transform infrared (FTIR) spectroscopic analysis
The spectra of gelatin samples were recorded using an FTIR-Spectrum, dried gelatin and KBr powder (1/100) (w/w) were mixed uniformly and pressed into the slice. The spectra were acquired IR range into the mount of 4,000–500 cm⁻¹(mid-IR region) at 25 °C. Prior to data analysis, the spectra were baseline corrected and normalized.

2.5. Amino acid analysis
Amino acid composition in bone gelatin was determined using UPLC (Ultra Performance Liquid Chromatography). A total of 0.1 g of sample is put into a screw tube, then 5 mL of 6 N HCl is added. This solution is hydrolyzed in an oven for 22 hours at 110 °C. The obtained hydrolyzate is cooled at room temperature, then dissolved in a 50 mL volumetric flask with aquabidest. After homogeneous, the solution is filtered with filter paper and then filtered again using a 0.2 nm filter then put into a tube. Next, a sample tube was filled with 500 µL of the filtered sample, 40 µL of AABA (standard amino acid), and 460 µL of aquabidest. The sample is vortexed and derivatized. Derivatization is carried out using micro vials which are filled with 10 µL of sample or standard, and 70 µL of borate buffer is then vortexed for 10 seconds. After that, the sample is left at room temperature for 1 minute then heated at a temperature of 55 ºC for 10 minutes. Then the microbial is inserted into the vial to be injected into the UPLC (Ultra-Performance Liquid Chromatography).

2.6. Statistical analysis
A completely randomized design was used, and all experiments were performed in triplicate. Data were compared using analysis of variance (ANOVA). Data were compared using, and the probability value of p<0.05 was considered significant. The analysis was performed using SPSS 21.
3. Results and Discussion

3.1. Gelatin yield
The yield of gelatin shown in table 1. Data on the yield of patin fish bone gelatin is normally distributed data ($p>0.05$) based on Kolmogorov-Smirnov analysis. ANOVA analysis results showed that the difference in extraction time affected the yield of gelatin ($p<0.05$). In this study, the gelatin yield was decreased at 7 hours extraction time (UB7), while according to [15] increasing extraction time will increase the yield. Gelatin yield increased at 5 hours of extraction time (UB5), 5.33 ± 1.03%. Increasing the extraction time provides more energy to disrupt the collagen structure stabilizing bond so that the helical transition becomes a coil [19].

This increase in yield is also influenced by the mechanical and cavitating effects of ultrasonic as time increases, so more samples are damaged, and gelatin is released gradually. This extraction process causes the breaking of hydrogen bonds between the three tropocollagen chains into 3 free chains (α chains), 2 linked chains (β chains) and 3 chains that are still bonded (γ chains) [20]. The low yield obtained from this study shows that the extraction method is still not really efficient, and the selection of appropriate gelatin sources and extraction optimization is needed.

The yield obtained was influenced by each gelatin extraction process, starting from the stage of decreasing, demineralization, washing, and extraction stage with hot water. In the demineralization stage, the use of acid concentration and the length of time of demineralization to become ossein will greatly affect the yield. The immersion time in the demineralization process is 48 hours to become ossein; this time is chosen after preliminary research. The time of soaking will affect the amount of collagen that is hydrolyzed, the longer the soaking time, the more collagen will be hydrolyzed. Excessive immersion time will cause further hydrolysis so that the gelatin will be degraded and the yield will decrease.

| Parameters          | UB 3          | UB 5         | UB 7          |
|---------------------|---------------|--------------|---------------|
| Yield (%)           | 3.04±0.57a    | 5.33±1.03b   | 3.06±0.83a    |
| Gel strength (Bloom)| 173.94±2.89c  | 147.74±0.83b | 74.1±7.22a    |
| Viscosity (cP)      | 10.6±0.10a    | 14.63±0.31b  | 30.27±0.79c   |

3.2. Gel strength
One of the important properties of gelatin is its ability to convert liquids to form solids or change the shape of soles into reversible gels so that the strength of the gel is an important characteristic to know the physical properties of gelatin. Gel strength measurement in this study uses grams of Bloom. The strength of the gel from the bone gelatin is presented in table 1. The results showed that the gel strength was in the range of 74.1-173.94 grams of Bloom.

From Table 1, the strength shows that the gel strength decreases when extraction time increases. The same thing was also obtained in the research of [20,15]. The strength of the gel is influenced by the molecular weight distribution and the amino acid chain content in gelatin resulting from the extraction conditions [14]. Longer ultrasonic extraction times provide greater energy to break the chain of amino acids to form shorter amino acid chains so that the gel strength becomes low [15].

It is based on [21,22] using catfish bone gelatin with extraction optimization obtained gel strength of 254.7% and 230.2%, respectively. Gelatin extracted from tuna fish [23,24] obtained the gel strengths are 88.74-133.91 and 167.8 Bloom, respectively. These difference between species are caused by different sizes of protein chains and complex interactions that are determined by amino acid composition.
According to [26], the specifications of the gelatin that can be used as edible gelatin range from 50 to 300 Bloom.

3.3. Viscosity

Viscosity is an important physical property of gelatin which determines the quality and application of gelatin in certain fields. Data of viscosity shown in table 1. From table 1 shows that the increasing extraction time, the viscosity value is also increasing. Based on the research of [8] the viscosity value of freshwater fish is associated with the high number of amino acids alanine, proline, hydroxyproline. This shows that the content of 3 amino acids above increases with increasing extraction time. However, the physical properties of gelatin not only depend on amino acid composition but also the presence of α, β, and γ chains, and the aggregates of high molecular weight molecules and protein fragments of low molecular weight molecules. A large number of β and γ chains have a negative impact on the functional properties of fish gelatin, such as low viscosity [28] but longer amino acid chains will higher the viscosity.

Gelatin viscosity values obtained ranged from 10.6 to 30.27 cP. This value is higher than the viscosity obtained from catfish bone species, namely 3.1 and 4.6 cP [23,24], catfish and patin bones are 4 - 11.3 cP and 34 cP [29,30]). This viscosity value was successfully improved from the viscosity obtained by [24] to close to the value obtained from [29]. This proves that ultrasonic can improve the physical properties of gelatin in the form of viscosity. According to [24], the viscosity value of gelatin recommended as edible gelatin is 15-75 cP.

3.4. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Molecular weight analysis of gelatin with SDS-PAGE is presented in figure 1 and approximation value of the molecular weight of different gelatin show in table 2 analyzed by Photocape software using a low range of marker (M) (97-14 KDa). The software will approximate the value of molecular protein weight. Molecular weight distribution includes the α1/α2 ratio and the number of β chains that determine the physical properties of gelatin, such as gel strength and melting point [5]. A decrease in the intensity of the α chain band occurs in gelatin with an extraction time of 7 hours (UB7), which has the lowest gel strength. Based on the research of [30], the effect of temperature and extraction time can affect the protein content of gelatin.

![Figure 1: The molecular weight of gelatin was analyzed by SDS-PAGE](image-url)
In the study of [15] comparing ultrasonic and water bath extraction methods found that the range of gelatin protein, molecular weight values from the ultrasonic method was lower compared to the water bath.

### 3.5. Amino acid composition

The selected amino acid gelatin composition is presented in table 3. According to [31] the composition of amino acids is very influential on the physical properties of gelatin produced. The main amino acids that make up gelatin are glycine, proline, and hydroxyproline. Based on table 3, the biggest amino acid content was glycine 24.4% then proline 12.3% and glutamic acid 11.18%. In this study, the analysis of hydroxyproline amino acids was not carried out because of the absence of standards for these amino acids. Based on research by [5] better viscosity was found in gelatin of warm water fish such as tilapia because of the high content of alanine, proline, and high hydroxyproline, while for cold water fish had a smaller container for the three amino acids. Gelatin amino acid content is similar to collagen with 2/3 parts of amino acids is glycine and the rest is proline and hydroxyproline.

Proline and hydroxyproline are amino acids found in collagen and gelatin. Hydroxyproline is involved in the formation of gels, which act as proton donors and hydrogen bonding can be formed if they are close to chains that have proton acceptors groups [18]. This is also confirmed from [33] research, presence of these amino acids is closely related to the ability of gelatin to bind with water because the hydroxyl group in hydroxyproline acts as a stabilizer of the helix by interacting hydrogen through bridges connected by water molecules to form hydrogen bonding with a carbonyl group.

### Table 3. Gelatin molecular weight.

| Samples | $\beta$ bands (KDa) | $\alpha_1$ bands (KDa) | $\alpha_2$ bands (KDa) |
|---------|---------------------|------------------------|------------------------|
| UB 3    | 155.82              | 128.03                 | 120.65                 |
| UB 5    | 155.82              | 128.60                 | 120.09                 |
| UB 7    | 158.09              | 128.60                 | 120.08                 |

### Table 3. Amino acid composition of selected gelatin.

| Amino acid       | Parameters | mg/g  |
|------------------|------------|-------|
| Essential        | Histidine  | 4.7352|
|                  | Threonine  | 22.2126|
|                  | Leucine    | 22.5767|
|                  | Lysine     | 38.6760|
|                  | Valine     | 18.8269|
|                  | Isoleucine | 11.378|
|                  | phenylalanine | 12.9723|
|                  | Tyrosine   | 4.3924|
|                  | Aspartic acid | 38.9889|
|                  | **Glycine** | **157.0699** |
|                  | Arginine   | 65.6266|
|                  | Alanine    | 65.62658|
|                  | Glutamic acid | 71.8859|
|                  | Serine     | 28.6201|
3.6. FTIR spectra analysis

Functional groups analysis with FTIR was performed on selected gelatin. Based on Figure 2, there is 5 important amide uptake which describes the characteristics of amino acids and the proportion of amino acids from gelatin, namely amides A, B, I, II, and III. Amide A is an N-H stretch which shows the presence of hydrogen bonds [34], observed at wave numbers 3437 cm$^{-1}$. Some free NH strain vibrations usually occur in the range 3400-3440 cm$^{-1}$. When the N-H group of the peptide is involved in hydrogen bonds it will shift to a low wave number, which is 3300 cm$^{-1}$ [26]. Amide B is observed at wave numbers 2927 cm$^{-1}$.

Amide I is the absorption band for strain vibration C=O along the polypeptide chain observed at wave number 1643 cm$^{-1}$. Amide I shows the confirmation of the polypeptide chain, different uptake depicting different conformational changes in proteins [15]. Based on the research of [15] which compared the ultrasonic method with no ultrasonic for the extraction of gelatin, in the amide, I by ultrasonic method gives greater intensity. This shows the loss of a larger molecular sequence due to the separation of cross-molecular bonds by ultrasonic use [21]. Amide II is observed at a wave number of 1521 cm$^{-1}$, this is due to the vibration of the bending of N-H joining the C-N strain, usually this amide II is observed at wave numbers 1550-1600 cm$^{-1}$. [34]. Amide II is a vibration caused by a combination of C-N strain and N-H breakdown in peptides [21].

Amide III represents variations in peaks between C-N strain and N-H formation from amide connections, as well as uptake arising from vibrations of -CH$_2$ groups of glycine and proline side chains [35]. This Amide III is observed in the wave number 1240 cm$^{-1}$. In the study of [34], this amide is observed at wave numbers 1232–1238 cm$^{-1}$. The absence of a shift of amide III to wavenumbers 1449 cm$^{-1}$, indicates the triple helical structure of gelatin is not maintained [26].

![Figure 2. FTIR spectra of selected gelatin.](image)

4. Conclusion

Extraction of gelatin from catfish bone by the ultrasonic method was successfully carried out and can use as alternative halal gelatin sources. Based on the physical and chemical properties of gelatin, UB 5 was chosen as the best treatment. The results of functional group analysis with FTIR vibrations which
showed amides A, B, I, II, and III were characteristic of gelatin, and the results of molecular weight analysis showed the bands β, α1, and α2. The physical and chemical properties of gelatin that have met the required standards. In this study, ultrasonic has succeeded in improving the physical properties resulting from gelatin such as gel strength and viscosity compared to previous studies.

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