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# Extraction Process Optimization and Functional Characteristics of by Papain Solubilized Collagen from Large Yellow Croaker (*Pseudosciaena crocea*) Skin

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**Abstract:** Through collagen extraction from large yellow croaker (*Pseudosciaena crocea*) skin using an enzymatic method, the extraction optimization and characterization of collagen from raw materials after papain hydrolysis pretreatment are studied. The extraction process was optimized using the response surface methodology in this study. The findings show that the optimal conditions are a ratio (solvent to solid) of 25.0 mL/g, a time of 42.0 h, a temperature of 40.0°C and a pH of 7.0. Under these optimum conditions, the collagen extraction rate is 66.17%. Based on the FTIR spectra analysis, collagen can maintain a high-interacted structure, and collagen peptides have a complete triple helix structure. In addition, the SEM analysis shows that the extracted collagen has well-defined fibril morphologies. Also, the foaming capacity and emulsifying ability of extracted collagen are superior to commercial type I collagen.

**Keywords:** Large Yellow Croaker Skin, Collagen, Extraction, Characteristics, Response Surface Methodology (RSM)

## Introduction

A vital component of the extracellular matrix, collagen is a structural protein with fibers (Maroušek *et al*., 2015). With almost 30% of all animal proteins being made up of it, it is the most common protein found in animals (Pati *et al*., 2010; Tang *et al*., 2022). The structural characteristics of collagen are established by chemical analysis, and the collagen molecule's most notable feature is its triple helix structure. Three polypeptide chains, which can be identical or different, make up the distinctive structure of collagen (Bhagwat and Dandge, 2018). Collagens have found widespread application as functional materials in the food, cosmetic, biomedical, and pharmaceutical industries (Ahmed *et al*., 2020).

The flavor and excellent nutritional value of the large yellow croaker (Larimichthys crocea) have led to its extensive culture in China (Hui *et al*., 2016). It is also among China's most valuable marine fish species in terms of the economy (Wu *et al*., 2018). An abundant fish species in the South and East China Seas, the large yellow croaker is a demersal warm-temperate fish. It can be processed to create many different culinary products, such as dried fish, salted fish, and smoked fish (Gao *et al*., 2013). There is a significant amount of by-product generated during processing. Large yellow croaker skin accounts for 3-5% of its body weight. Its skin can be processed into high value-added product, especially collagen.

Since collagen is insoluble in its natural state, it can be extracted with the help of subsequent pretreatments. Most of the collagen used in commercial products are considered mammals, including pigs and cows. However, collagen from mammalian sources is facing problems, such as hazard of disease, infection, and spiritual barriers. Because of this, there has been a rise in demand this year for realistic collagen obtained from other sources, particularly those that have an aquatic origin (Sinthusamran *et al*., 2013). Compared to other sources, the yield derived from aquatic sources is higher. Therefore, it is obvious that collagen from fish can be utilized as an alternative for safer, more environmentally friendly, and more consumer-friendly collagen (Ali *et al*., 2018). Collagen obtained from fish scales displays fantastic absorption, superb biocompatibility, biodegradability and low antigenicity (Muthumari *et al*., 2016). Numerous studies have revealed the highly soluble collagens are come from a variety of aquatic sources, including fish scales, fins, bones, and skin (Arumugam *et al*., 2018; Kiew *et al*., 2014;

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Gaurav Kumar *et al*., 2015). The extraction of collagen and the biological activity of peptides from collagen produced from marine food waste were reviewed by Ahmed *et al*. (2020). Pretreatment of raw materials and extraction of collagen are two main processes in the collagen extraction process. Pretreatment's primary goal is to enhance the acquired collagen's quality by eliminating contaminants (Ahmed *et al*., 2020). Several different methods are used for extracting collagen, such as ultrasound assisted extraction, enzyme, acid, and salt extraction (Lu *et al*., 2023; Tacias-Pascacio *et al*., 2021). Because of its high extraction efficiency, enzymatic extraction is a promising technique for collagen extraction, which can reduce the antigenicity of collagen and have no effect on the collagen triple helix structure. The extraction rate varies greatly from 0.09-80%, and most of them were lower than 50% (Ahmed *et al*., 2020). Collagen has different chemical properties and many functional properties related to their protein side chain groups, such as emulsifying and foaming. It is difficult to find the source and preparation method of collagen with high extraction efficiency and good functional characteristics. To extract antioxidant peptides from the gelatin of Pacific cod (*Gadus macrocephalus*) skin, a number of enzymes, including alkalase, trypsin, pepsin, neutrase, and papain, were assessed. Papain hydrolysate showed the best antioxidant activity (Tacias-Pascacio *et al*., 2021). The functional properties and hydrolysis optimization of the collagens recovered by papain from large yellow croaker skin are not studied, despite the fact that there are researches on the collagen extraction from marine sources using enzymatic extraction methods. To this end, this study aims to prepare collagen from large yellow croaker skin using papain and characterize it. Papain hydrolysis is optimized for collagen extraction using the response surface technique. Furthermore, the extracted collagen's foaming and emulsifying properties are assessed and contrasted with those of commercial type I collagen. The findings may offer a foundation for the commercially manufacturing of producing collagen from aquatic sources.

## Materials and Methods

### *Experimental Materials and Chemicals*

We bought large yellow croakers (*Pseudosciaena crocea*) in Zhanjiang, Guangdong, China, from a local aquatic market. They weighed an average of 0.8-1.0 kg. The fish were killed and dissected upon arrival at the lab. Subsequently, the skins underwent descalement and a distilled water wash. Lastly, two days before collagen extraction, the skins were cleaned and then sliced into tiny pieces (1×1 cm) and stored frozen at-18°C.

Commercial papain (20,0000 u/g, pH6.0-7.0, 55-65°C) was purchased from Aladdin reagent (Shanghai) Co., Ltd. Analytical grade was the minimum for all other substances and reagents for this study.

### *Extraction of Collagen*

The collagen was extracted through the method of Zhang *et al*. (2016); Dhakal *et al*. (2018) with some modifications.

To eliminate non-collagenous proteins and colors, 2 g of skin samples were treated with NaOH (0.1 m, 1:10, w/v) and continuously stirred for 1 h with a magnetic stirrer. The fish skin samples were then rinsed until pH neutral with distilled water. Cyclohexane was applied to the skin for 6 h in order to eliminate fat, and they were frozen at -18°C before the collagen was extracted. Collagen was extracted from the defatted skins using papain enzymatic hydrolysis. The solvent/solid ratio, temperature, time and pH were selected as the variables. Papain (2%, w/v) was used in an enzymatic hydrolysis process that was carried out with gentle stirring at various temperatures (35, 40, 45, 50 and 55 ºC), periods (32, 36, 40, 44 and 48 h), pH values (4, 5, 6, 7 and 8), and solvent/solid ratios (10, 15, 20, 25 and 30 mL/g). Then, the solution was filtered to eliminate the solids, and the filtrate was recovered. The solution was analyzed as a papain soluble collagen preparation. Every experiment was run in triplicate. Collagen was precipitated, dialyzed and freeze-dried and subsequently lyophilized into flakes for further analysis using the freeze-dryer (Bo Kang Lab equipment medical Co., Ltd., Beijing, China).

### *Determination of extraction yield*

With minor adjustments, the extraction solution's hydroxyproline content was measured citing the method outlined by Ali *et al*. (2018). First, the 0.5 mL extracted collagen solution was hydrolyzed with hydrochloric acid (6 m) for 12 h at 120-130°C and clarified using activated carbon. After that, paper made of Whatman filter was used to filter it and the pH was brought to almost neutral by adding 0.1 m NaOH. After adding the oxidant solution (0.1 mL) and isopropanol (0.2 mL) to the neutralized sample (0.1 mL) in a test tube, all reagents were well combined. The oxidant solution was made up of acetate/citrate buffer (pH 6) and chlororamine T (7%, w/v). Finally, Ehrlich's reagent (1.3 mL): p-dimethylamino-benzaldehyde (2 g) combined with perchloric acid (3 mL, 60%, w/v) and isopropanol ratio was incorporated (3:13, (v/v). The mixture was combined, and for 20 min, it was incubated at 60°C in a water bath. Before measuring the absorbance at 558 nm, 5 mL of isopropanol were added to the reaction and it was cooled in running water. Using hydroxyproline standards ranging from 0.5-2.5 g/mL, a hydroxyproline curve was created. Using the standard curve, the hydroxyproline content was calculated.

With a few minor adjustments, the hydroxyproline content of the large yellow croaker skin was ascertained using the method published by Kittiphattanabawon *et al*. (2005). The skin was hydrolyzed for 24 h at 115°C methanesulfonic acid (4.0 m) with 3-2(2-aminoethyl) indole (0.2%, v/v) under reduced pressure. After neutralizing the hydrolysates with NaOH (3.5 m), they were diluted using citrate buffer (0.2 m, pH 2.2). An amino acid analyzer (A300, membraPure Co., Germany) was used to receive an aliquot of 0.4 mL.

The yield (%) of collagen extracted from large yellow croaker (*Pseudosciaena crocea*) skin was calculated according to Eq. (1):

$$Extraction Yield\,(\%) = He \times 100 / Hs \quad (1)$$

where, *He* and *Hs* are the amount of hydroxyproline content from the extracted solution and the large yellow croaker skin, respectively.

### *Experimental Design for Collagen Extraction*

RSM is a statistical tool used to identify the optimal combination of variables for the intended response and construct an empirical model. The collagen extraction process from large yellow croaker (*Pseudosciaena crocea*) skin was optimized by the designed expert software. A response pattern was identified and a model was created using Box Behnken Design (BBD) with four variables: Hydrolysis time ($X_1$), ratio of solvent/solid ($X_2$), temperature ($X_3$), and pH ($X_4$) (Arumugam *et al*., 2018). Based on the outcomes of first single-factor trials, the levels and independent factors were selected. The dependent variable in this case was the yield of collagen extraction. Table 1 presents several layers of independent variables. Three replicates were used for each of the 29 runs that were carried out at the central point. The model of a full quadratic equation is represented as predicting the optimal point with the help of the following equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_i X_i^{\,2} + \sum \beta_{ij} X_i X_j$$

where, $X_i$, $X_i^2$ and $X_j$ denote the individual, quadratic impacts, and interactive of the variables on the response result, respectively, and $Y$ is the response variable that reflects the collagen extraction yield. The regression coefficients are $\beta_0$, $\beta_i$, $\beta_{ii}$ and $\beta_{ij}$.

### *UV-Vis Absorption Spectra*

The UV absorption spectra of collagen were calculated using the methodology of Sampath Kumar *et al*. (2012) with minor adjustments. After dissolving the sample in 10 mL of 0.5 m acetic acid, 10 mg of the sample was added to a collagen solution, which was then centrifuged at 8,000 g for 15 min at 4°C. The absorbance of supernatant was determined with a UV-visible spectrophotometer in wavelength scanning mode at various wavelengths (190-400 nm).

### *Fourier Transform Infrared Spectroscopy*

Analysis was done on the prepared collagen's chemical fingerprint. Using the KBr compression pellet method, samples of freeze-dried collagen and standard type I collagen were generated. They were then scanned between 500 and 4000 $cm^{-1}$ wavenumber using a step resolution of 2 $cm^{-1}$. (Sampath Kumar *et al*., 2012). The prepared samples were subjected to a Nicolet 6700 FT-IR equipment (thermo fisher scientific, USA).

### *Scanning Electron Microscopy (SEM)*

The prepared collagen's surface appearance and microstructure were examined using SEM (JSM-7610F, JEOL Ltd., Japan). The sample was adhered to SEM electro conductive paste and gold particles were vacuum-coated onto the sputter. The coated samples were placed inside SEM's specimen chamber and their surface appearance was investigated. The 8 kV accelerating voltage was used for the SEM observations. Commercial type I collagen served as a control.

### *Foaming Properties of Extracted Collagen*

With minor adjustments, the approach of Koli *et al*. (2012) was used to determine the foaming properties. 50 mL of distilled water and 0.25 g of collagen powder were combined, and then the solution was homogenized for 3 min at room temperature at 7400 rpm. The fluid was quickly poured into a graduated 100 mL cylinder, and the foam's volume was recorded. The foam stability was assessed after 30 min by monitoring the foam's volume drop.

Equations (2-3) were used to compute *FC* and *FS*, respectively:

$$FC\,(\%) = (V_0 - 50) \times 100\% / 50 \quad (2)$$

$$FS\,(\%) = (V_{30} - 50) \times 100 / (V_0 - 50) \quad (3)$$

where, $V_0$ is the volume at 0 min after whipping, and $V_{30}$ is the total volume left at 30 min.

### *Emulsifying Properties of Extracted Collagen*

With minor adjustments, the emulsification characteristics of the sample were ascertained using the methodology outlined by GAO *et al*. (2023). Following a 3:1 (v/v) ratio of maize oil and the prepared collagen sample (10 mg/mL), the solution was homogenized for 1 min at 10,000 rpm. After pipetting the emulsion (50 μL)

out of the mixture, a 0.1% sodium dodecyl sulfate solution was added in 5 mL. Lastly, *ESI* and *EAI* were measured using the absorbance at 500 nm respectively, using Eqs. (4-5):

$$EAI\,(m^2/g) = 2 \times 2.303 \times A_0 \times N / (10000 \times \psi \times c) \quad (4)$$

$$ESI = 10 \times A_0 / (A_0 - A_{10}) \quad (5)$$

where, $N$ is the dilution coefficient; $c$ is the sample concentration; $A_0$ represents the absorbance at 0 min, $A_{10}$ represents the absorbance at 10 min, and $\psi$ the oil volume percentage in the emulsion.

### *Statistical Analysis*

Every experiment was run three times, and the three sets of experimental data were used to compute the standard deviations. The RSM regression models were fitted to the experimental data to produce tri-dimensional and contour graphs. A comprehensive analysis of variance was conducted in terms of the coded level of variables in order to examine the impact of each individual variable. A significance level of $p<0.05$ is applied to the difference between the average values.

## Results and Discussion

### *Effect of Hydrolysis Time on Extraction Yield of Collagen*

An extension of the hydrolysis time (32-48 h) was used to maximize the extraction process's hydrolysis while keeping other variables constant. Figure 1a illustrates how the yield of collagen extraction rises with increasing time, particularly when the period was between 32 and 40 h. An additional marginal improvement was noted when the duration surpassed 40 h. At 48 h, the maximum collagen extraction yield from fish skin was 68.73%. Consequently, the ideal hydrolysis period was determined to be between 40 and 48 h. According to Mohammadi *et al*. (2016), the ideal hydrolysis time for the collagen soluble in pepsin-containing eggshell membrane was shown to be within the range of 36-48 h. After 48 h of hydrolysis, solvent saturation resulted in a drop in PSC extraction rate and enzyme activity. Collagen releases at a time-dependent rate into the bulk extraction medium from the matrices skin. The length of time needed for collagen to be exposed to the release medium during hydrolysis may have an impact on extraction efficiency since papain can breakdown collagen and cause it to diffuse out of the material (Mohammadi *et al*., 2016).

### *Effect of Solvent/Solid Ratio on Extraction Yield of Collagen*

Another significant factor influencing the collagen extraction efficacy is the ratio of solvent to solid. Figure 1b illustrates how the solvent/solid ratio affects collagen extraction yield. An increased yield of collagen extraction from large yellow croaker (*Pseudosciaena crocea*) skin may result from increasing the solvent/liquid to solid ratio. When the ratio exceeds 25 mL/g, the extraction yield of collagen decreases gradually. In addition, there is an optimal value (71.55%) of solvent/solid ratio when obtaining the highest collagen extraction yield. The possible reason may be that as the solvent increases, the collagen in fish skin could full contact with the papain and be hydrolyzed. However, when the amount of solvent exceeds a certain value, both the papain and collagen are diluted due to the increased solvent. According to Kiew *et al*. (2014), the collagen particles' concentration gradient and velocity of diffusion into solution from the large yellow crowker skins rose at a larger solvent/solid ratio, improving the extraction's efficiency.

### *Effect of Temperature on Extraction Yield of Collagen*

The effect of temperature on the extraction yield of collagen from large yellow croaker (*Pseudosciaena crocea*) skin is shown in Fig. 1c. There is an optimal value (51.86%) of temperature (40°C) to get the largest collagen extraction yield. Collagen extraction yield would be reduced if this temperature were greater or lower. The possible reason is that papain has its optimal temperature, and lower or higher temperature can lead to the decrease of the enzyme activity or collagen damage. As a result, at an optimum temperature, papain can produce the best hydrolysis effect and obtain better extraction efficiency. Therefore, the optimal reaction temperature for extracting collagen from large yellow croakers by papain hydrolysis is 40°C.

### *Effect of pH on Extraction Yield of Collagen*

The extraction effect of pH (4-8) on collagen is assessed while maintaining the same values for the other three variables, as shown in Fig. 1d. When the pH value is between 4 and 7, as the pH increases, the collagen extraction yield from large yellow croaker skin increases gradually. The highest extraction yield of collagen (68.32%) was at a pH of 7, this is because the enzyme activity was strong under this condition. When the pH value exceeded or below 7, the extraction rate decreased. Therefore, the optimal pH value for extracting collagen from large yellow croaker by papain hydrolysis is 7.

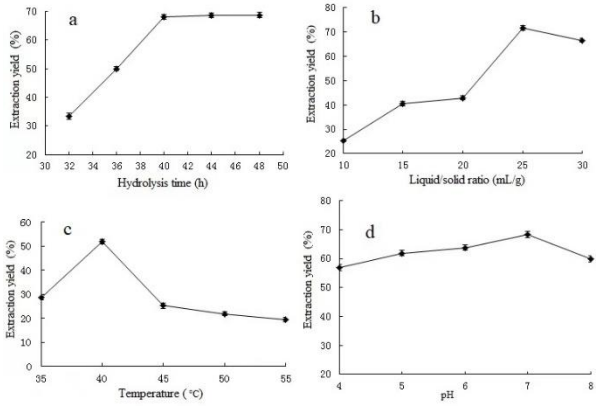


**Fig. 1:** Effect of hydrolysis time, solvent/solid ratio, temperature and pH on extraction yield of collagen

**Table 1:** Coded values and independent variables used for extraction optimization

| | | Coded factor level | | |
|---|---|---|---|---|
| Independent variables | Symbol | -1 | 0 | +1 |
| Hydrolysis time (h) | $X_1$ | 36 | 40 | 44 |
| Solvent/solid ratio (mL/g) | $X_2$ | 20 | 25 | 30 |
| Temperature(ºC) | $X_3$ | 35 | 40 | 45 |
| pH | $X_4$ | 6 | 7 | 8 |

## *Optimization of Collagen Extraction Parameters*

RSM is a helpful technique for assessing how different factors affect the responses, which can be employed to optimize the extraction conditions (Tokay *et al*., 2022). RSM analyzes the response data from the collagen extraction process by various combined conditions in order to identify the ideal extraction parameters (Table 1). A total of 29 runs are displayed in Table 2, along with their BBD experiment responses. The collagen extraction rates in Table 2 range from 35.34-72.55%. A second-order polynomial equation represents the empirical relationship between the independent variables and responses that was obtained by applying multiple regression analysis to the experimental data:

$$Y = 70.32 + 0.40X_1 + 0.98X_2 + 1.17X_3 + 0.39X_4 + 2.57X_1X_2 + 0.90X_1X_3 + 0.97X_1X_4 + 1.45X_2X_3 - 0.75X_2X_4 - 0.40X_3X_4 - 16.86X_2^2 - 15.46X_3^2 - 13.06X_4^2$$

where, $Y$ is the extraction yield of collagen; the hydrolysis time, solvent/solid ratio, temperature, and pH are represented by the coded variables $X_1$, $X_2$, $X_3$, and $X_4$.

**Table 2:** Box-Behnken design and experimental observation for the extraction yield of collagen

| Run order | Hydrolysis time (h) $X_1$ | Solvent/solid ratio (mL/g) $X_2$ | Temperature (ºC) $X_3$ (%) | pH $X_4$ | Collagen extraction Yield (%) |
|---|---|---|---|---|---|
| 1 | 0(40) | +1(30) | -1(35) | 0(7) | 39.07±0.68 |
| 2 | 0(40) | +1(30) | 0(40) | +1(8) | 45.61±0.57 |
| 3 | -1(36) | 0(25) | 0(40) | +1(8) | 39.43±0.73 |
| 4 | +1(44) | 0(25) | 0(40) | +1(8) | 41.09±0.98 |
| 5 | 0(40) | -1(20) | 0(40) | -1(6) | 37.86±0.32 |
| 6 | 0(40) | +1(30) | 0(40) | -1(6) | 44.14±0.59 |
| 7 | +1(44) | +1(30) | 0(40) | 0(7) | 41.06±0.62 |
| 8 | 0(40) | 0(25) | 0(40) | 0(7) | 72.55±0.73 |
| 9 | 0(40) | -1(20) | 0(40) | +1(8) | 42.32±0.48 |
| 10 | 0(40) | 0(25) | 0(40) | 0(7) | 70.98±0.63 |
| 11 | 0(40) | +1(30) | +1(45) | 0(7) | 43.37±0.74 |
| 12 | +1(44) | 0(25) | +1(45) | 0(7) | 41.87±0.76 |
| 13 | -1(36) | +1(30) | 0(40) | 0(7) | 36.92±0.71 |
| 14 | -1(36) | 0(25) | -1(35) | 0(7) | 35.34±0.63 |
| 15 | +1(44) | 0(25) | 0(40) | -1(6) | 40.36±0.72 |
| 16 | 0(40) | -1(20) | +1(45) | 0(7) | 39.75±0.39 |
| 17 | 0(40) | 0(25) | +1(45) | -1(6) | 43.11±0.47 |
| 18 | +1(44) | 0(25) | -1(35) | 0(7) | 37.22±0.56 |
| 19 | +1(44) | -1(20) | 0(40) | 0(7) | 35.55±0.61 |
| 20 | 0(40) | 0(25) | 0(40) | 0(7) | 70.64±0.73 |
| 21 | 0(40) | 0(25) | 0(40) | 0(7) | 69.40±0.61 |
| 22 | 0(40) | -1(20) | -1(35) | 0(7) | 41.25±0.57 |
| 23 | 0(40) | 0(25) | 0(40) | 0(7) | 68.03±0.67 |
| 24 | -1(36) | 0(25) | +1(45) | 0(7) | 36.39±0.47 |
| 25 | 0(40) | 0(25) | -1(35) | +1(8) | 40.93±0.65 |
| 26 | 0(40) | 0(25) | -1(35) | -1(6) | 39.52±0.55 |
| 27 | 0(40) | 0(25) | +1(45) | +1(8) | 42.91±0.51 |
| 28 | -1(36) | -1(20) | 0(40) | 0(7) | 41.68±0.68 |
| 29 | -1(36) | 0(25) | 0(40) | -1(6) | 42.58±0.47 |

**Table 3:** Regression analysis of variance of predicted quadratic model

| Source | Sum of squares | Degrees of freedom | Mean square | F-value | p-value | Remark on significance |
|---|---|---|---|---|---|---|
| Model | 3845.15 | 14 | 274.65 | 78.25 | <0.0001 | Significance |
| $X_1$ | 1.91 | 1 | 1.91 | 0.55 | 0.4725 | |
| $X_2$ | 11.53 | 1 | 11.53 | 3.28 | 0.0914 | |
| $X_3$ | 16.51 | 1 | 16.51 | 4.70 | 0.0478 | |
| $X_4$ | 1.87 | 1 | 1.87 | 0.53 | 0.4773 | |
| $X_1X_2$ | 26.36 | 1 | 26.36 | 7.51 | 0.0159 | |
| $X_1X_3$ | 3.26 | 1 | 3.26 | 0.93 | 0.3516 | |
| $X_1X_4$ | 3.75 | 1 | 3.75 | 1.07 | 0.3187 | |
| $X_2X_3$ | 8.43 | 1 | 8.43 | 2.40 | 0.1436 | |
| $X_2X_4$ | 2.23 | 1 | 2.23 | 0.64 | 0.4388 | |
| $X_3X_4$ | 0.65 | 1 | 0.65 | 0.19 | 0.6733 | |
| $X_1^2$ | 1844.74 | 1 | 1844.74 | 525.59 | <0.0001 | |
| $X_2^2$ | 1359.09 | 1 | 1359.09 | 387.22 | <0.0001 | |
| $X_3^2$ | 1550.05 | 1 | 1550.05 | 441.63 | <0.0001 | |
| $X_4^2$ | 1107.11 | 1 | 1107.11 | 315.43 | <0.0001 | |
| Residual | 49.14 | 14 | 3.51 | | | |
| Lack of fit | 37.55 | 10 | 3.76 | 1.30 | 0.4319 | Not significant |
| Pure error | 11.58 | 4 | 2.90 | | | |
| Corrected total | 3894.28 | 28 | | | | |

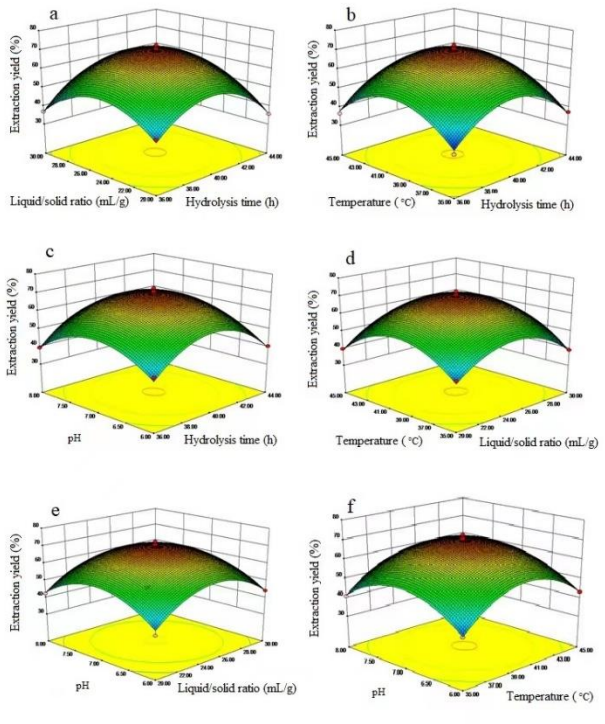


**Fig. 2:** Response surface plot with the effects of hydrolysis time, solvent/solid ratio, temperature and pH on collagen extraction

Table 3 displays the result of analysis of variance of the quadratic model for collagen extraction yield. The model's non-fitting value, 0.4319, is not significant at the level of 95% confidence, but the model's p-value is less than 0.0001, indicating significance (Table 3). It is verified that the tested model's fitting is efficient and ideal based on these two parameters. The coefficient of determination ($R^2$) is a measure of fitness and can fully represent the connection between the responses and the measured independent variables in real systems (Norziah *et al*., 2013). A low $R^2$ score suggests that the model's dependent variables are not very relevant (Wang *et al*., 2008). $R^2$ value also determines the high significance of the model through variance analysis, indicating strong agreement between the estimated and experimental collagen extraction yield values.

The 2D contour plots of two independent variables and two replies' 3D response surface plots depict the regression quadratic equations for the two responses (Norziah *et al*., 2013). The interactions between the four measured variables and the experimental levels can be illustrated by visual methods. Figure 2 shows how the yield of collagen extracted from large yellow croaker skin is affected by temperature, pH, solvent/solid ratio, and hydrolysis time. Each plot in Fig. 2 presents the influence results of two factors, while the other two are at the center point of the experiment range. 2D contour plots of Fig. 2 are all elliptical, indicating the interactions between corresponding variables cannot be ignorable.

A 3D surface and contour plot of the combined effects of hydrolysis time and solvent/solid ratio on the collagen extraction yield is shown in Fig. 2a. The extraction yield of collagen increases initially and subsequently falls with solvent/solid ratio (20-25 mL/g) and time (36-40 h), reaching the maximum at 40 h and 25 mL/g. Figures 2b-f made with the other variables show a similar pattern. As the time increases, the collagen extraction yield increases until about 40 h and then decreases in Fig. 2b. At the temperature of about 40°C, the collagen extraction yield reaches the peak. The impact of hydrolysis time and pH on the collagen extraction effect is displayed in Fig. 2c,

which reaches the maximum predicted value at 40 h and pH 7. It is evident that at 40°C and 25 mL/g, the collagen extraction yield reaches its maximum in Fig. 2d. It can be seen from Fig. 2e that the extraction yield reaches the maximum predicted value at 25-28 mL/g and pH 7. Figure 2f illustrates how temperature and pH affect the collagen extraction yield, which reaches the maximum predicted value at 39-41°C and pH 7.

### *Validation of the Model*

The calculating tool indicates that 42.34 h, a temperature of 40.29°C, a pH of 7.03 and a solvent/solid ratio of 25.44 mL/g are the ideal parameters for getting the maximum collagen extraction yield. The extraction yield of collagen at these ideal conditions is 65.06%. According to approximately optimal conditions, the predicted results are verified in triplicate. For precise control of the parameters, the optimum time is modified to 42.0 h, the optimum solvent/solid ratio to 25.0 mL/g, the optimum temperature to 42.0°C and the optimum pH to 7.0. After modification, the yield of collagen extraction is 66.17%. Within the 95% confidence interval, the predicted and experimental values do not differ significantly. The yield of pepsin-solubilized collagen were 26.67, 46.6 and 60.3% for the skins of Malaysian catfish, for grass carp, for bighead carp, respectively (Kiew *et al*., 2014; Zhang *et al*., 2007; Liu *et al*., 2012). The results in our experiments are higher than those of above references. The types of enzymes, raw materials, extraction conditions, and extraction process preparation techniques are all thought to be responsible for the variation in yield.

The solvent/solid ratio of 25.0 mL/g, the hydrolysis time of 42.0 h, the temperature of 42.0°C, and the pH of 7.0 are found to be the ideal parameters for achieving the maximum extraction yield of papain-soluble collagen. The maximum collagen yield of 66.17% of skin is achieved under the optimal conditions. With an $R^2$ value of 0.9814, the derived mathematical model shows good generalization and consistency between the expected and experimental values of the collagen extraction yield.

### *UV Absorbance*

Figure 3 displays the isolated collagen sample's UV absorption spectrum. The sample's maximum absorption peak is less than 300 nm, and the observation is at approximately 235 nm. This is in line with collagen's characteristic that the usual range for the absorption peak is 220-280 nm. At 230 nm, the triple-helix collagen peak may be found (Kaewbangkerd *et al*., 2023; Sampath Kumar *et al*., 2012). The collagen polypeptide chain's groups C=O, -COOH, and $CONH_2$ may be connected to the maximum absorbance (Gaurav Kumar *et al*., 2015). It is worth noting that for 280 nm, there is no other maximum absorption peaks. This can be explained by the low levels of tyrosine in the collagen samples, which exhibit a maximal absorption peak at 280 nm (Pezeshk *et al*., 2022). Hence, the results indicate that the extracted collagen has a high purity, and the non-collagenous protein have been removed efficiently, indicating there is collagen. Similar results were observed when collagen was isolated from the skins of catla and rohu (Gaurav Kumar *et al*., 2015) and from the bones of two marine fish (Sampath Kumar *et al*., 2012).

### *FTIR*

From the infrared spectrum, it can be seen whether the collagen helix structure is well maintained. The FTIR spectra of two samples are displayed in Fig. 4. The amide A peaks of the prepared collagen and control are at 3389 and 3406 $cm^{-1}$, respectively. As a result of hydrogen bonds' free N-H stretching vibration, amide A usually occurs at wavenumber (3400-3440 $cm^{-1}$) (Muthumari *et al*., 2016). The amide A peak of the extracted collagen is lower than that of the control, suggesting that more of the protein's N-H groups establish intermolecular hydrogen bonds and move to a lower wavenumber. The asymmetric stretch of $CH_2$ is connected to the amide B peaks of the collagen sample and the control, which are located at 2924 and 2927 $cm^{-1}$, respectively. The sample and control have amide I peaks measured at 1653 and 1648 $cm^{-1}$, respectively. Due to the stretching vibrations of the N-H bond and the C=O, the characteristic wavenumber of amide I occurs in the 1600-1700 $cm^{-1}$ range (Pezeshk *et al*., 2022). Generally, Amide II is the cause of the combination of N-H bending and C-N stretching. Amide II bands can be detected at 1543 and 1542 $cm^{-1}$ for the sample and control groups, respectively. The N-H deformation and C-N stretching elements present in the amide III peak give rise to the triple helix structure of collagen (Muthumari *et al*., 2016). The sample's and the control's amide III absorption peaks in this investigation are located at 1245 and 1240 $cm^{-1}$. Furthermore, the absorption ratio between the $CH_2$ bend and the amide III band is nearly 1.0, suggesting that the collagen samples' triple helix conformation is still stable. The collagen structure is highly conserved, according to the FTIR data, and it resembles the typical type I collagen structure.

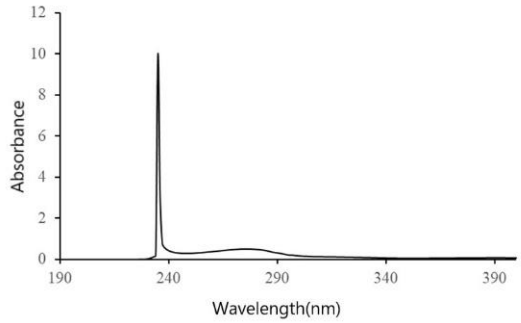


**Fig. 3:** UV absorption spectrum of extracted collagen sample

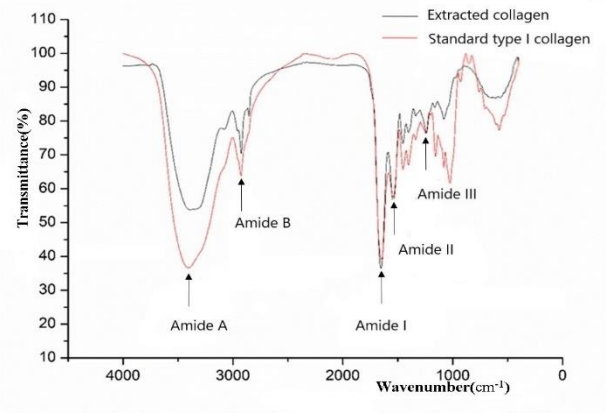


**Fig. 4:** FTIR spectra of extracted collagen and standard collagen

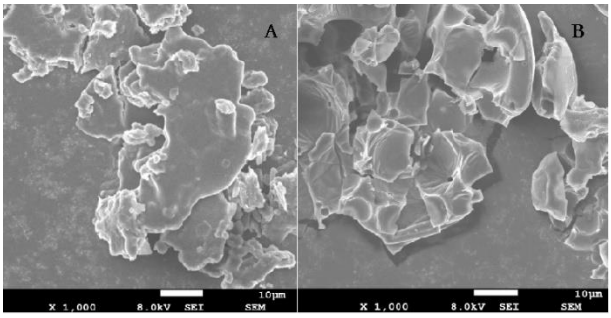


**Fig. 5:** SEM of the collagen (A: Standard type I collagen; B: Extracted collagen)

**Table 4:** Foaming properties and emulsifying properties of extracted collagen

| Functional property | FC (%) | FS (%) | EAI ($m^2/g$) | ESI (min) |
|---|---|---|---|---|
| Extracted collagen | 55.34±1.24[a] | 62.57±1.48[a] | 64.72±1.14[a] | 12.65±0.64[a] |
| type I collagen | 50.12±1.07[b] | 68.24±1.13[b] | 53.85±1.18[b] | 15.37±0.44[b] |

Note: The table's value is the mean plus standard deviation. Significant variations in therapy were indicated by different letters in each column ($p<0.05$)

### *Scanning Electron Microscopy*

Collagen's microstructure and morphological traits can be observed via SEM (Fig. 5). It shows that the extracted collagen has a complex and irregular flake fibril form, which is typical for purified collagen. Moreover, the extracted sample exhibits a thick, asymmetrical structure, and its somewhat wrinkled surface may be related to the dehydration that occurred during the freeze-drying procedure (Pezeshk *et al*., 2022). The findings indicate that the extracted collagen has no major structural changes with standard type I collagen. However, the tuna skin's acid-soluble collagen microstructure underwent notable physical alterations following ultrasonic treatment (Pezeshk *et al*., 2022).

### *Foaming Properties*

Table 4 displays the isolated collagen's foaming capacity and stability, which are 55.34±1.24% and 62.57±1.48%, respectively. Although the findings of foaming stability differ, the extracted collagen has a significantly ($p<0.05$) higher foaming capacity than commercial type I collagen. The FC of soybean protein isolate is about 50% (Li *et al*., 2011), which is lower than that of the prepared collagen and standard type I collagen. Collagen's ability to foam is influenced by its source, extraction method, and soluble protein content. Collagen foam production is usually inhibited by the transportation, penetration, rearrangement, and redistribution of collagen molecules at the interface of air-water. The type of collagen film that forms determines the stability of foaming (Koli *et al*., 2012).

### *Emulsifying Properties*

Table 4 also displays the isolated collagen's stability and emulsifying capabilities. The extracted collagen's emulsifying activity and stability indexes are 64.72±1.14% and 12.65±0.64%, respectively, while those of commercial type I collagen are 53.85±1.18% and 15.37±0.44%, respectively. Compared with commercial type I collagen, the emulsifying capacity of extracted collagen is significantly ($p<0.05$) higher, but the emulsifying stability did not follow the same pattern. The EAI of soybean protein isolate is about 45% (Li *et al*., 2011), which is lower than that of extracted collagen and standard type I collagen. Collagen's emulsifying qualities in the food industry are intimately linked to its surface features. Collagen has good foaming and emulsifying properties related to its surface behaviors, which could be explained by the hydrophobic and charged groups found in the collagen structure's side chains. The source and the extraction method have a significant impact on these functional characteristics of collagen (Tang *et al*., 2022).

Emulsifying and foaming qualities are crucial functional characteristics of proteins (Han *et al*., 2024). The application scope can be expanded by verifying collagen's emulsifying and foaming qualities. The findings show that the extracted collagen has good emulsifying and foaming properties.

## Conclusion

According to this results, RSM can be utilized to describe and forecast the collagen extraction from large yellow croaker skin by papain hydrolysis. The ideal conditions are identified to maximize the collagen extraction yield. The maximum amount of collagen that the skin can produce under ideal conditions is 66.17%. This study can improve the current industry of isolating intact collagen, but there are some limitations in practical applications, such as incubation with papain for 42 h and enzyme stability. This problem might be resolved by using immobilized papain.

The extracted collagen has functional properties such as emulsifying and foaming. Generically, large yellow croaker skin can supply high-quality collagen that is similar to type I collagen that is sold commercially. The results are helpful for collagen extraction from large yellow croaker skin by enzymatic method and chemical characteristics. However, further studies on the biological functions (such as ACE-I inhibitory, antitumor, antioxidant, antibacterial and anti-freezing activities etc.,) of this collagen as a new resource are needed.

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## Author's Contributions

**Biaoshi Wang and Wenfeng Li:** Designed, performed the experiments, written the drafted and revised the manuscript.

**Shumin Liu and Xiaojun Hu:** Engaged in the process of gathering experiment-related supplies and did some experiments.

**Shiqi Zhang and Shengyuan Yang:** Analyzed the experimental data and revised the manuscript.

## Ethics

None of the authors of this article have conducted any animal studies. The authors assume full responsibility for any ethical dilemmas that may emerge following the manuscript's publication.

### *Conflict of Interest*

The authors assert that they do not have conflicting agendas. The co-author certifies that every author has perused and given their approval to the manuscript.

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