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Box-Wilson Design and Analysis for Extraction of Gelatin from Black Kingfish Skin

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Abstract

Gelatin is a derived, protein-based biopolymer used in nourishments to enhance consistency, strength, and flexibility. The aim of this research is to optimize the organic acid extraction of gelatin from fish skin. In this work, effect of parameters such as acid (based on its type) concentration (0.05-0.15 M), extraction temperature (40-60 °C), and types of acid (formic, acetic and propionic acids) on extraction yield of gelatin was examined at constant extraction time of 7 d and skin to acid mass ratio of 1:20. Collagenous material was separated from fish skin, and a basic protein, collagen, was hydrolyzed to gelatin, a derived protein. Response surface methodology (RSM) based Box-Wilson design was used for optimization and the optimization results revealed that the maximum gelatin extraction of 11.91% was achieved at 0.1 M formic acid and 52 °C. Thus, it would be concluded that the fish skin could be the potential feedstock for gelatin extraction.

Keywords

Acid extraction
Collagen
Fish skin
Gelatin
Optimization

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Introduction

Gelatin, a derived protein, is an unadulterated protein nourishment fixing, attained by the warm denaturation of a basic protein, collagen (Ling, 2014). Gelatin is a water-soluble proteinaceous substance arranged by forms, which includes the demolition of the tertiary and the essential structure of collagens by the partial hydrolysis of collagen obtained from the skin, white connective tissues and bones of animals (Appell *et al.*, 2018; Das *et al.*, 2021). Also, it is a high molecular weight polypeptide and a significant hydrocolloid, which has demonstrated for its applications in a wide scope of nourishment items, to a great extent, due to its gelling and thickening properties (Oberoi *et al.*, 2019). It varies from different hydrocolloids, on the grounds, that the greater part of them is polyacrylamide; however, gelatin is a palatable protein containing all the fundamental amino acids except for tryptophan (McClements *et al.*, 2009). The amino corrosive organization, especially regarding proline and hydroxyproline, can fluctuate from species to species, because of presentation to a wide scope of ecological conditions, especially temperature (Saxer, 2010; Tiwari & Srivastava, 2012). Cow bones, pig skins, and fishes are the primary sources of gelatin. Numerous nourishments use gelatin as hotspot for surface and restricting operator (Gómez-Guillén *et al.*, 2011). Cheow *et al.* (2007) studied the gelatin extraction from skin of sin croaker and shortfin scad using 0.3% (w/v) H_2SO_4 and 0.7% (w/v) citric acid, and finally with water at 45 °C overnight. Nalinanon *et al.* (2008) investigated the utilization of bigeye snapper skin for gelatin extraction using 0.025 M NaOH (1:10) at room temperature for 2 h followed by soaking with 0.2 M acetic acid (1:10) in the presence of pepsin for 2 days at 4 °C in the presence of trypsin inhibitor, and finally with water 45 °C for 12 h.

Xu *et al.* (2011) examined the extraction of gelatin from *Amur sturgeon* skin with 0.2 M H_3PO_4 (1:10) at 4 °C for 24 h, and finally with distilled water (50 °C, 1:5 w/v) for 6 h. Duan *et al.* (2011) utilized carp skin for gelatin extraction with10% (v/v) butyl alcohol (1:10) at 4 °C, and finally with distilled water (1:15 w/v) for 4 h at 60, 70 and 80 °C. Killekar *et al.* (2012) yielded 13.88% gelatin from black kingfish skin through extraction with 0.2% NaOH followed by 0.2% H_2SO_4 and 1% citric acid with skin to solvent mass ratio of 1:7, and finally with water at mass ratio of 1:3 and 45 °C for 12 h. Sila *et al.* (2015) yielded 6.82% gelatin from the skin of barbel fish by extraction with 0.05 M NaOH followed by the addition of pepsin for 48 h. Zhang *et al.* (2016) obtained a yield of 80.6-93.1% on dry basis from skin of tilapia and channel catfish through extraction with 0.2% H_2SO_4 followed by the addition of 0.2%

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citric acid, and finally with water at 45 °C for 12 h. Díaz-Calderón *et al.* (2017) utilized salmon skin for gelatin extraction with 0.05 M acetic acid at 60 °C. Silva *et al.* (2017) yielded 19.7% gelatin from kumakuma fish skin by extraction with 0.3 M NaOH, and finally with distilled water at 58 °C for 6 h. Zhang *et al.* (2019) investigated the utilization of spiny dogfish skin for gelatin extraction using alcalase for 2.1 h, and finally with water at 50 °C for 46 h.

Based on the literature review, in this research, black kingfish skin was selected as a substrate for gelatin extraction. Response surface methodology is a statistical optimization tool based on the development of quadratic models involving independent factors with two types of designs-Box-Wilson or central composite and Box-Behnken designs (Bidgoli *et al.*, 2010). Skin was washed thoroughly to remove debris, then soaked with NaCl to remove non-collagen, and then with organic acid followed by distilled water to obtain gelatin (Fan *et al.*, 2017). The objectives of the present are: (i) To characterize black kingfish skin for its biochemical composition; and (ii) To optimize gelatin extraction from black kingfish using different organic acids, acid concentrations and extraction temperature using response surface methodology.

Materials and methods

Chemicals

Black kingfish skin was collected from the local fish market. Formic, acetic, and propionic acid, procured from VWR international, were used in this study without further processing. Double distilled water was used in this research, unless specified.

Preparation of fish skin

Fish skin was manually removed at the market and immediately packed in polythene bag, kept in ice with a skin/ice ratio of 1:3 (w/w) in a polystyrene box, and transported to the laboratory in an hour. Upon arrival, residual meat was removed manually from the skin and washed with tap water. The skins were cut into pieces (22x12 cm) with scissors and placed in polythene bags. The skins were stored at -20 °C for further use.

Biochemical characterization of fish skin

Fish skin was characterized for chemical composition such as protein, lipid, carbohydrate, moisture, and ash. Protein, lipid, carbohydrate, moisture, and ash were determined by Lowry's method (Lowry *et al.*, 1951), Soxhlet extraction (Soxhlet, 1879), anthrone method (Hodge & Hofreiter, 1962), hot air oven method (AOAC, 2005b) and furnace method (AOAC, 2005a). All the reported methods for characterization of fish skin were standard operating procedures reported by Sadasivam and Manickam (1992).

Removal of non-collagenous proteins

The frozen fish skin was thawed at 4 °C overnight in the refrigerator before use. Non-collagenous proteins from the skin were removed by stirring in chilled water at 5 °C for 10 min. 10 g of skin was weighed, and the washing was performed with skin to water mass ratio of 1:6. The water washing was repeated twice. Then, the skin was mixed with 0.8 M NaCl at 5 °C for 10 min in the same mass ratio of 1:6 and washed with double distilled water. The salt washing was repeated thrice for the removal of non-collagenous material.

Extraction of gelatin from collagen

The extraction step was conducted by gently stirring the mixture of collagenous material and acid in the mass ratio of 1:20 at the different temperatures (40-60 °C) and concentrations of acid (0.05-0.15 M) for 24 h. The wet collagen was placed in a hot air oven at 60 °C for 24 h. The moisture content was determined, and water was added to adjust the moisture content to 15%. Then, the material was placed in a hot air oven again at 45 °C for 30 min for the purification of gelatin. All the experiments were performed in triplicate and the mean value was reported as a response. Extraction yield was calculated based on the mass of fresh fish skin, as given in Eq. (1).

$$Extraction\ yield\ (\%) = \frac{Mass\ of\ gelatin\ obtained}{Mass\ of\ fresh\ fish\ skin\ used} x100 \quad (1)$$

Statistical optimization of gelatin extraction by response surface methodology-based Box-Wilson design

Response surface methodology develops quadratic model with minimum trials and error. Also, it is used to study the interaction between independent factors (Sivamani *et al.*, 2020). It has two designs: Box-Wilson or central composite design and Box-Behnken design. According to their principle, number of experiments performed for Box-Wilson design is $2^f + 2 \times f + m$ and $2 \times f \times (f\text{-}1) + m$ for Box-Behnken design, where f is number of independent factors and m is number of centre points (Joaquin *et al.*, 2021; Sivamani & Baskar, 2018). Normally, Box-Wilson design requires more experiments and produces accurate results than Box-Behnken design.

An original version of Design-Expert 13 from Stat-Ease, Inc. USA, was used to perform the response surface design and analysis. Box-Wilson design is used in this research to maximize extraction yield at optimum values of acid concentration (0.05-0.15 M), extraction temperature (40-60 °C), and types of acid (formic, acetic, and propionic acids). The ranges were selected based on literature Zhang *et al.* (2019)). In this study, type of acid is considered as a categoric factor and extraction temperature and acid concentration as numeric factors. All the experiments were performed as per the Box-Wilson design matrix, and the experimental data was tested to fit linear, 2FI (two factor interactive), quadratic and cubic models for their significance. Then, the data was fitted to the significant quadratic equation to investigate the effect of independent factors on the response was represented in Eq. (2) and model coefficients were evaluated using Eq. (3). After evaluation, the coefficients were substituted in Equation (2) for checking the deviation between experimental and predicted values. The significance of the model was tested using analysis of variance (ANOVA) based on high *F*-value and low *p*-value with 95% confidence level (Sivamani *et al.*, 2020). Also, regression coefficient R^2, difference between adjusted and predicted R^2, and predicted residual error sum of squares (PRESS) values were also assessed to find the goodness-of-fit between experimental and predicted values (Vijayanand *et al.*, 2021).

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j + \varepsilon \quad (2)$$

$$\beta = (X^T X)^{-1}(X^T Y) \quad (3)$$

Where: β_o is an intercept; β_i, β_{ii}, β_{ij} are linear, squared and interaction coefficients respectively, Y is the response; X_i and X_j are independent factors; and ε is a random error.

Results and discussion

Biochemical characterization of fish skin

On an average, total weight of black kingfish is 1.75 kg, out of which body weight is 1.6 kg, head weight is 0.15 kg, and skin weight is 75 g. Fish skin was characterized for chemical composition, and found to contain 20.68% protein, 1.12% lipid, 0.44% carbohydrate, 76.49% moisture and 1.27% ash on wet basis.

Statistical optimization of gelatin extraction by response surface methodology-based Box-Wilson design

Table (1) shows the independent factors and levels selected for extraction of gelatin from skin of fresh black kingfish. Since independent factors is a mixture of numeric and categoric factors, number of experiments performed for Box-Wilson design is $(2^f+2\times f+m)\times k$ where f is number of independent numeric factors, k is number of levels in independent categoric factors, and m is number of centre points. Therefore, number of experiments performed was $(2^2+2\times2+3)\times3=33$.

Table (2) shows the Box-Wilson matrix of 33 experiments for extraction of gelatin from skin of black kingfish. Table (3) shows the testing of various models, viz., linear, 2FI, quadratic and cubic models for the extraction of gelation from the skin of black kingfish. The values of *F*, *p*, R^2, difference between adjusted and predicted R^2 and PRESS were used to check the significance of model. A high *F*-value of 77.93, low *p*-value of <0.05, $R^2>0.8$, difference between adjusted and predicted $R^2<0.2$ and a least value of PRESS of 14.35 for quadratic model implied that it is significant and suitable for the extraction of gelatin from black kingfish skin.

Table 1. Independent factors and levels used for gelatin extraction from black kingfish skin

Independent factor	Symbol	Unit	Levels		
			Low (-1)	Centre (0)	High (+1)
Concentration of acid	A	M	0.05	0.10	0.15
Extraction temperature	B	°C	40	50	60
Type of acid	C	-	Formic	Acetic	Propionic

Table 2. Box-Wilson design matrix for gelatin extraction from black kingfish skin

Std	Run	A: Concentration (M)	B: Temperature (°C)	C: Type of acid	Extraction yield (%)	
					Experimental	Predicted
22	1	0.1	50	Acetic	9	9.12
20	2	0.1	50	Acetic	9.25	9.12
18	15	0.1	40	Acetic	8	3.63
12	17	0.05	40	Acetic	7	5.97
15	18	0.15	60	Acetic	7	4.97
17	20	0.15	50	Acetic	8	7.64
13	21	0.15	40	Acetic	6	5.22
16	23	0.05	50	Acetic	7	9.63
19	25	0.1	60	Acetic	8	8.30
21	27	0.1	50	Acetic	8.75	11.85
14	33	0.05	60	Acetic	6	5.88
3	8	0.05	60	Formic	10	11.85
2	9	0.15	40	Formic	8	10.30
9	10	0.1	50	Formic	12	6.47
10	12	0.1	50	Formic	11.75	8.07
7	13	0.1	40	Formic	10	5.47
4	19	0.15	60	Formic	9	6.40
6	22	0.15	50	Formic	11	6.73
5	24	0.05	50	Formic	10	9.63
8	26	0.1	60	Formic	11	7.61
11	30	0.1	50	Formic	12.25	6.40
1	32	0.05	40	Formic	9	10.01
24	3	0.15	40	Propionic	4	7.28
29	4	0.1	40	Propionic	6	10.34
23	5	0.05	40	Propionic	5	8.07
33	6	0.1	50	Propionic	8	11.30
26	7	0.15	60	Propionic	5	9.12
25	11	0.05	60	Propionic	6	7.64
27	14	0.05	50	Propionic	6	7.22
28	16	0.15	50	Propionic	5	11.85
32	28	0.1	50	Propionic	8.25	7.64
30	29	0.1	60	Propionic	7.75	8.96
31	31	0.1	50	Propionic	6.75	6.07

Table 3. Testing of various models for gelatin extraction from black kingfish skin

Source	Sum of Squares	df	Mean Square	*F*-value	*p*-value	R^2	Adj. R^2	Pred. R^2	PRESS
Linear	103.13	4	25.78	15.13	< 0.0001	0.6837	0.6385	0.5624	66.01
2FI	2.98	5	0.5958	0.3063	0.9041	0.7034	0.5873	0.2550	112.38
Quadratic	39.43	2	19.71	77.93	< 0.0001	0.9648	0.9463	0.9049	14.35
Cubic	2.93	8	0.3656	1.99	0.1297	0.9842	0.9610	0.8756	18.76

Table 4. Analysis of variance (ANOVA) for quadratic model developed for gelatin extraction from black kingfish skin

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	145.53	11	13.23	52.30	< 0.0001	significant
A-Concentration	0.5000	1	0.5000	1.98	0.1744	
B-Temperature	2.53	1	2.53	10.01	0.0047	
C-Type of acid	100.09	2	50.05	197.85	< 0.0001	
AB	0.3333	1	0.3333	1.32	0.2639	
AC	1.33	2	0.6667	2.64	0.0952	
BC	1.31	2	0.6563	2.59	0.0984	
A^2	21.22	1	21.22	83.90	< 0.0001	
B^2	8.32	1	8.32	32.88	< 0.0001	
Residual	5.31	21	0.2530			
Lack of Fit	3.77	15	0.2514	0.9783	0.5518	not significant
Pure Error	1.54	6	0.2569			
Cor Total	150.84	32				

The experimental data was fitted to the quadratic model, as represented in Eq. (4), (5) and (6) for formic, acetic, and propionic acids, respectively. All the interactive and quadratic terms are same, irrespective of type of acid. The changes are observed only in linear terms. An increase in negative coefficient terms tends to decrease the response and vice versa. Similarly, a decline in positive coefficient terms tends to diminish the dependent factor and vice versa (Chandrasekaran & Sivamani, 2018). In Eq. (4), (5) and (6), positive values of coefficients in linear terms and negative values of coefficients in quadratic terms tend to maximize the response, i.e., gelatin extraction. All the data points lie within ±10% of predicted and experimental values, which agrees with the statistical inference.

$$Y = -21.48983 + 113.68421 \times A + 1.06272 \times B + 0.333333 \times A \times B - 668.42105 \times A^2 - 0.010461 \times B^2 \quad (4)$$

$$Y = -22.38377 + 120.35088 \times A + 1.01272 \times B + 0.333333 \times A \times B - 668.42105 \times A^2 - 0.010461 \times B^2 \quad (5)$$

$$Y = -25.65271 + 107.01754 \times A + 1.07522 \times B + 0.333333 \times A \times B - 668.42105 \times A^2 - 0.010461 \times B^2 \quad (6)$$

Table (4) shows the analysis of variance (ANOVA) for quadratic model developed for the extraction of gelatin from the skin of black kingfish. From the ANOVA table, it is evident that the model was significant with high *F*-value of 52.30 and low *p*-value of < 0.05. Also, acid concentration, and interactive terms were not significant with p-value > 0.05. All the other linear terms like extraction temperature and type of acid, and quadratic terms are significant with *p*-value < 0.05. This means that type of acid is more important factor that affects extraction yield than extraction temperature followed by acid concentration (Alipal *et al.*, 2021). Also, it is evident that standard deviation of response values is 0.5029 which is less than ±5%. Coefficient of variance (C.V.) of 6.25% implied that the experimental values are in accordance with the predicted values.

Effect of process parameters on extraction yield

The effect of process parameters such as acid concentration, extraction temperature and type of acid on extraction yield for extraction from black kingfish, is shown in Fig. (1). The acid concentration was varied from 0.05 to 0.15 M by keeping extraction temperature, time, solid to acid mass ratio at 50 °C, 24 h, and 1:20 respectively, irrespective of type of acid. When the acid concentration varied from 0.05 to 0.10 M, extraction yield increased from 10 to 12.25%. When the acid concentration further enhanced to 0.15 M, extraction yield decreased to 11%. So, it is evident that the optimal region is located around 0.1 M to achieve maximum extraction yield. After 0.1 M acid concentration, gelatin started to denature due to solubilizing of bonds between amino acids in gelatin. The extraction temperature was varied from 40 to 60 °C by keeping acid concentration, time, solid to acid mass ratio at 0.1 M, 24 h, and 1:20 respectively. When the extraction temperature varied from 40 to 50 °C, extraction yield has been increased from 10 to 11.75%. When the temperature further enhanced to 60 °C, extraction yield has been decreased to 11%. So, it is evident that the optimal region is located around 50 °C to achieve maximum extraction yield. After 50 °C, due to increase in temperature, the gelatin structure started to break down because, at higher temperatures, intra- and intermolecular bonds are broken as the amino acid molecules gain more kinetic energy. Formic, acetic, and propionic acids were varied by keeping acid concentration, extraction temperature, time, solid to acid mass ratio at 0.1 M, 50 °C, 24 h, and 1:20 respectively. The yield of gelatin for extraction using formic, acetic, and propionic acids from black kingfish skin were 12, 9 and 8% respectively. Due to addition of methylene group to lower acids, the gelatin structure started to denature.

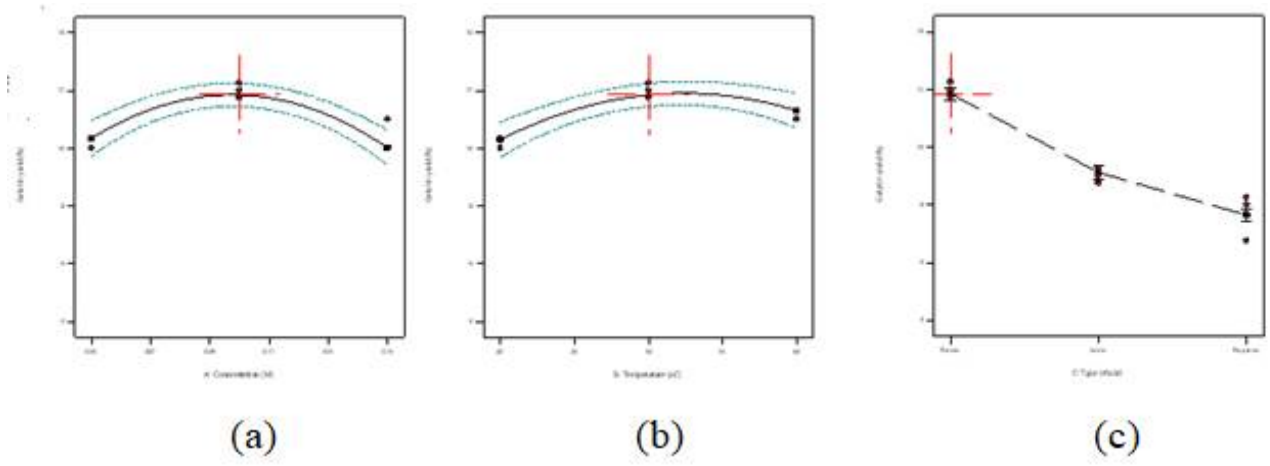

Fig. 1. Effect of (a) acid concentration, (b) extraction temperature and (c) type of acid on extraction yield from black kingfish.

Fig. (2) shows the 3D interactive effect between acid concentration and extraction temperature for formic acid. The interactive effect was studied by varying acid concentration from 0.05 to 0.15 M and extraction temperature from 40 to 60 °C for formic acid. An interactive plot shows the same pattern of variation for acetic and propionic acids. The extraction yield of 8.95% was obtained at a lowest acid concentration and extraction temperature of 0.05 M and 40 °C, respectively. At this point, when the acid concentration increased to 0.10, the extraction yield enhanced to 10.30%; further increment of acid concentration to 0.15 M leads to decrease in extraction yield to 9.63%.

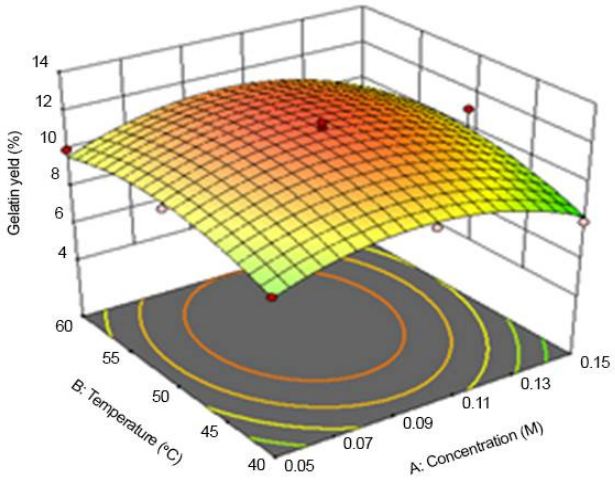

Fig. 2. Interactive effect of acid concentration and extraction temperature on extraction yield from black kingfish for formic acid.

Similarly, the extraction yield increased to 10.34% at the acid concentration and extraction temperature of 0.05 M and 50 °C, respectively. The extraction yield decreased to 8.29% when the extraction temperature further increased to 60 °C at the same acid concentration of 0.05 M. Also, the extraction yield of 11.85 and 10% were obtained at extraction temperatures of 50 and 60 °C at the acid concentration of 0.10 M. Finally, the extraction yield of 11.33 and 9.63% were observed at extraction temperatures of 50 and 60 °C at the acid concentration of 0.15 M. From the interactive effect between acid concentration and extraction temperature for formic acid, the optimal conditions were found to be acid concentration and extraction temperature of 0.10 M and 52.3 °C, respectively to obtain extraction yield of 11.91%.

Conclusion

The presented work aimed to maximize the yield of gelatin extracted from black kingfish skin by varying organic acids (formic, acetic, and propionic), acid concentration (0.05-0.15 M) and extraction temperature (40-60 °C). The optimal values show that the maximum extraction yield of 11.91% was achieved at acid concentration and extraction temperature of 0.10 M and 52.3 °C, respectively. The results showed the best goodness of fit between experimental and predicted values by Box-Wilson design. Thus, the black kingfish skin could be utilized as a cost-effective substrate for the extraction of gelatin.

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Conflict of interest

The authors declare that there is no conflict of interest.

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