OPTIMIZATION OF COLLAGEN EXTRACTION PROCESS FROM SKIN OF STINGRAY (*RAJIFORMES*)

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ABSTRACT

Collagen is widely used in the manufacture of food, pharmaceutical and cosmetic products. Collagen from fish skin has many advantages over other collagen sources, hence, the research of collagen from stingray skin is of interest. The process of cleaning and extracting collagen from stingray skin has been investigated and optimized to create good collagen quality from stingray skin. The optimal conditions of collagen extraction were determined, with three independent factors: acetic acid, solid-to-liquid ratio and extraction time which all affect the extraction efficiency of stingray skin. The optimal conditions to obtain the highest extraction efficiency were determined at acetic acid content of 0.578M, solid-to-liquid ratio of 1/11, and extraction time of 53 hours. Under these optimal conditions, the extraction efficiency reached the highest level of 72.29%. This result is the scientific basis for selecting the optimal conditions for collagen processing from the stingray skin byproducts with the highest quality, creating collagen with white color, and soft fiber structure to meet the commercial collagen quality.

Keywords: *collagen; stingray skin; extracted; process optimization; acetic acid.*

1. INTRODUCTION

Collagen is a major protein of extracellular matrix and connective tissue. Collagen accounts for 30% of total protein of the body and plays a key role in the composition of connective tissue such as skin, tendons, bones, ligaments [3, 13]. Collagen is widely used in food, cosmetic, medicine and pharmaceutical industries.

In food technology, collagen is used as an additive to improve gel durability, gel hardness of sausage, surimi, sausage film, beef wrap, as a functional food to help treat arthritis, support muscle growth, regenerate bone structure, and improve cardiovascular activity [1, 14].

In the past, collagen was mainly produced from the skin and bones of cows and pigs. However, from 2000 to the present, due to the outbreak of BSE (Bovine Spongiform Encephalopathy), and foot and mouth disease, collagen extraction studies have focused on alternative sources of aquatic origin [5, 6].

In Vietnam, there are a lot of by-products from seafood [8]. Of which, rays are a widely distributed species both nearshore and offshore, with relatively high production of rays, estimated at 11,500 tons per year. Rays have a high skin and cartilage content, which is a good source of collagen extraction.

2. MATERIAL AND METHODS

2.1 Materials

This study was conducted at the Biochemistry Laboratory and Food Laboratory 1, Faculty of Chemical and Food Technology, Ho Chi Minh City University of Technology and Education. The main material was rays from Vung Tau seafood market which was frozen and transferred to the laboratory for the research

2.2 Methods

2.2.1 Extraction of collagen

The process of collagen extraction from ray skin was conducted by chemical method. After being washed, the skin of fishes was cleaned with NaOH to remove non-protein impurities such as lipids, minerals…,etc. and to deodorize with H_2O_2. To achieve the purpose of purification, it is necessary to study the appropriate immersion of NaOH and H_2O_2. Collagen in fish skin was extracted with acetic acid to separate cross-linking between molecules and inter-molecules [8, 15]. The extract solution after centrifugation was precipitated by NaCl, then centrifuged, precipitated, dried, and crushed to obtain collagen.

2.2.2 Optimization of extraction process [2, 7]

Using an experimental planning method according to the rotation method of Box and Hunter with the objective function y (%) is the collagen extraction efficiency, the influencing factors are acetic acid concentration, extraction time and fish skin-to-extract solution ratio.

The quadratic regression equation is as below:

$$y = \hat{y} = b_0 + \sum_{1 \le j \le k} b_j x_j + \sum_{1 \le j \le i \le k} b_{ji} x_j x_i + \sum_{1 \le j \le k} b_{jj} x^2{}_j \quad (1)$$

Use the empirical planning method to find the coefficients of the equation (1).

The number of experiments was determined as:

$$N = 2^k + 2.k + n_0 N = 2^k + 2.k + n_0 \quad (2)$$

The arm of the matrix is defined as:

$$\alpha = 2k/4 \quad (3)$$

The formula to convert a real variable into a virtual variable is as follows:

$$x_j = \frac{z_j - z_j^0}{\Delta z_j} x_j = \frac{z_j - z_j^0}{\Delta z_j} \quad (4)$$

The parameters of the empirical equation are tested using the Student standard. Student coefficient of b_j coefficients is determined as below:

$$t_j = \frac{|b_j|}{s_{bj}} t_j = \frac{|b_j|}{s_{bj}} \quad (5)$$

The compatibility of mathematical models compared with experimental data is tested by Fisher standard:

$$F = \frac{s_{tt}^2}{s_{th}^2} F = \frac{s_{tt}^2}{s_{th}^2} \quad (6)$$

After determining the function form, optimizing by using each alternate method is conducted to verify the experimental results.

2.2.3 Analysis method

The collagen content in the sample was determined by colorimetric method [9, 12]. The Erlich which has color with hydroxyproline was used then UV-VIS spectrophotometer was used to determine collagen content in the sample.

Some properties of the finished collagen were determined by chemical analysis methods, HPLC high performance liquid chromatography method according to TCVN 8764: 2012, SEM capture method, SDS-PAGE electrophoresis method and molecular structure determination according to Hulmes D.J., 2002 [4]

2.2.4 Data processing method

Using the mathematical tools with the support of Microsoft Excel 2010, Matlab programming V.7.01 and was proven in practice.

3. RESULTS AND DISCUSSION

3.1 Develop an optimal problem

The objective function y (%) is the collagen extraction efficiency and the influencing factors are acetic acid concentration, extraction time, fish skin-to-extract solution ratio (k = 3). The secondary quadratic regression equation is as below:

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{12} x_1 x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2$$

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{12} x_1 x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2 \quad (7)$$

The experiment was conducted according to the levels of influencing factors as Table 1.

Table 1. *Levels of influencing factors*

Factor	Levels					Interval variable, $\Delta\Delta x_i$
	-α	Lower level -1	Central level 0	Upper level +1	+ α	
x_1	0.16	0.3	0.5	0.7	0.84	0.2
x_2	27.82	36	48	60	68.18	12
x_3	6.64	8	10	12	13.36	2

Number of experiments was N = 20.

α = 1,682.

Conduct experiments according to the matrix of experimental planning orthogonal level 2 with three elements, experimental results were presented in Table 2.

Table 2. *Experimental planning matrix*

Number of experiments		Real variable			Coding variable			Objective function
N		Z_1 (M)	Z_2 (hour)	Z_3 (w/v)	X_1	X_2	X_3	y (%)
2^k	1	0.7	60	12	1	1	1	67.47
	2	0.3	60	12	-1	1	1	63.81
	3	0.7	36	12	1	-1	1	64.00
	4	0.3	36	12	-1	-1	1	65.36
	5	0.7	60	8	1	1	-1	68.82
	6	0.3	60	8	-1	1	-1	61.47
	7	0,7	36	8	1	-1	-1	64.28
	8	0,3	36	8	-1	-1	-1	54.36
2k	9	0.84	48	10	1.682	0	0	63.66
	10	0.16	48	10	-1.682	0	0	55.01
	11	0.5	68.18	10	0	1.682	0	67.09
	12	0.5	27.82	10	0	-1.682	0	53.46
	13	0.5	48	13.36	0	0	1.682	69.65
	14	0.5	48	6.64	0	0	-1.682	57.20
n_0	15	0.5	48	10	0	0	0	71.94
	16	0.5	48	10	0	0	0	71.19
	17	0.5	48	10	0	0	0	72.99
	18	0.5	48	10	0	0	0	68.53
	19	0.5	48	10	0	0	0	72.01
	20	0.5	48	10	0	0	0	67.02

The coefficients in equation (7) were calculated using the formula:

$$b_0 = a_1\sum_{i=1}^{N} y_i - a_2\sum_{j=1}^{k}\sum_{i=1}^{N} x^2_{ji}y_i \qquad (8)$$

$$b_j = a_3\sum_{i=1}^{N} x_{ji}y_i \ , j = 1 \div k \qquad (9)$$

$$b_{j1} = a_4\sum_{i=1}^{N} x_{ji}x_{1i}y_i \ , j,1 = 1 \div k; j \neq 1$$

$$b_{jj} = a_5\sum_{i=1}^{N} x^2_{ji}y_i + a_6\sum_{j=1}^{k}\sum_{i=1}^{N} x^2_{ji}y_i - a_7\sum_{i=1}^{N} y_i \qquad (10)$$

Processing data by Excel, the coefficients in equation (7) were calculated as follows:

Table 3. *Coefficients in equation (7)*

b_0	b_1	b_2	b_3	b_{12}
70.421	2.498	2.671	2.390	0.306
b_{13}	b_{23}	b_{11}	b_{22}	b_{33}
-1.871	-1.216	-3.276	-2.943	-1.829

The reproducible variance and the variances of the systems were calculated by the formula:

$$s^2_{th} = \frac{\sum_{j=1}^{n_0}(y_i - \bar{y}_{ij})^2}{n_0 - 1} \ ; \ \bar{y}_{ij} = \frac{\sum_{j=1}^{n_0} y_{ij}}{n_0}$$
(11)

$$s^2_{b_0} = a_1 s^2_{th};\ s^2_{bj} = a_3 s^2_{th};\ s^2_{bjl} = a_4 s^2_{th};\ b^2_{b_{jj}} = (a_5 + a_6)s^2_{th}$$
(12)

Student coefficient of b_j and t_j coefficients was determined as follows:

$$t_j = \frac{|b_j|}{s_{bj}} t_j = \frac{|b_j|}{s_{bj}}$$
(13)

The results were presented as Table 4.

Table 4. *Student coefficients of b_j and t_j*

t_0	t_1	t_2	t_3	t_{12}

74.395	3.977	4.254	3.806	0.373
t_{13}	t_{23}	t_{11}	t_{22}	t_{33}
2.280	1.482	5.357	4.813	2.992

For $f_2 = f_{th} = n_0\text{-}1 = 6\text{-}1 = 5$ and $p = 0.05$ in the Student table, t_p (f2) was found = $t_{0.05}$ (5) = 2,571. $t_0, t_1, t_2, t_3, t_{11}, t_{22}, t_{33} > t_p(f_2)$ so the coefficients $b_0, b_1, b_2, b_3, b_{11}, b_{22}, b_{33}$ in table 3 were meaningful. $t_{12}, t_{13}, t_{23} < tp$ (f2), so the coefficients b_{12}, b_{13}, b_{23} had no meaning.

The regression equation described for collagen extraction efficiency was as below:

$$\hat{y} = 70.421 + 2.498x_1 + 2.671x_2 + 2.390x_3 - 3.216x_1^2 - 2.943x_2^2 - 1.829x_3^2 \quad (14)$$

Check the compatibility of the regression line equation

With:

$f_{du} = N - 1 = 20 - 7 = 13$

$f_1 = f_{tt} = f_{du} - f_{th} = N - 1 - (n_0 - 1) = 13\text{-}5 = 8$

We have:

$$S_{du} = \sum_{j=1}^{N}(y_j - \hat{y}_j)^2 = 143{,}8595$$

$$S_{du} = \sum_{j=1}^{N}(y_j - \hat{y}_j)^2 = 143{,}8595$$

$$S_{th} = \sum_{j=1}^{n_0}(y_j - \bar{y}_{IJ})^2 = 26.94$$

Compatibility variance:

$$S^2_{tt} = \frac{S_{du} - S_{th}}{f_1} = 14.61494$$

Determining the Fisher standard:

$$F = \frac{s^2_{tt}}{s^2_{th}} = 2.712$$

Look up tables F1-p(f1, f2) = F0,95(8.5) = 4.818 > F = 2.712.

Thus, the empirical regression equation is compatible with the experimental data. Therefore, this equation can be used to describe and calculate collagen recovery efficiency from ray skin

3.2 Building the single objective optimization

From the regression equation for extracting collagen from ray skin (14), with:

$0.3 \le x_1 \le 0.7$

$36 \le x_2 \le 60$

$8 \le x_3 \le 12$

Need to find solutions so that:

$$X^{opt} = (x_1^{jopt}, x_2^{jopt}, x_3^{jopt}) \in\in \Omega\Omega_x \quad (15)$$

With: (-1.682 ≤≤ x1, x2, x3 ≤≤ +1.682)

To $y_{max} = y(x_1^{1opt}, x_2^{1opt}, x_3^{1opt})$

Using the method of alternating each variable with support from Matlab software and Excel 2010, we have:

Step 1: Choose a starting point $X^{(0)} = (-1, -1, -1)$

Choose $\varepsilon_y > 0$, $\varepsilon_x > 0$, $\varepsilon = 0{,}001$.

Determine the target function value at the starting point:

$y^{(0)} = y\,(x_1^{(0)}, x_2^{(0)}, x_3^{(0)}) = 65{,}36$.

Step 2: Fixed 2 variables and change 1 variable.

Session 1: fixed 2 variables x2 and x3 respectively were -1 and -1, let x1 run in the value domain (-1; 1) with 0.001 running step. Then y was the best at y (* 1) = y (0.388; -1; -1) = 61.07.

Session 2: Fixed x1 = 0.388 and x3 = -1, allowing x2 to run in the value domain (-1.1) with step 0.001. Then y was the best at y (* 2) = y (0.388; 0.445; -1) = 67.29.

Session 3: Fixed x1 = 0.388 and x2 = 0.454, for x3 running in the value domain (-1,1) with step 0.001. Then y was the best at y (* 3) = y (0.378; 0.445; 0.665) = 72.29. After 3 sessions, conclude that the best value y = 72.29 at x1 = 0.388; x2 = 0.454; x3 = 0.665 then ymax = 72.29. Or:

$x_{opt} = (0.388; 0.454; 0.653)$

Calculate real value from virtual value based on the formula: $Z_j = Z_j^0 + x_j.\Delta Z_j$

Then, the concentration of acetic acid was 0.578M; extraction time was 53 hours;

The ratio of solution-to-skin of fish was 11/1 or the ratio of fish skin-to-solution of acetic acid was 1/11 (w/v). Under these conditions, the collagen recovery efficiency reaches the maximum value of 72.29%.

Step 3: Check stop conditions

$$|y^{(1)} - y^{(0)}| = |72.29 - 65.36| = 6.93 > \varepsilon_y$$

$$|y^{(1)} - y^{(0)}| = |72.29 - 65.36| = 6.93 > \varepsilon_y$$

Unsatisfied with stopping conditions, we continue the second round: Select $X^{(1)}$ = (0.388; 0.445; 0.665) as the new starting point, then $y^{(1)}$ = 72.29. Continue to do as round 1, finally $y^{(2)}$ = 72.29. Check stop conditions:

$$|y^{(1)} - y^{(0)}| = |72.29 - 72.29| = 0 < \varepsilon_y$$

$$|y^{(1)} - y^{(0)}| = |72.29 - 72.29| = 0 < \varepsilon_y$$

Satisfying the stopping condition, we could conclude that y reached the optimal value at:

X(1) = (0.388; 0,454; 0,653)

With ymax = 72.29 %.

3.3 Verify the experimental results

To re-evaluate the experimental results, the verification at the above optimal value was conducted. The results obtained were completely consistent with the experiment and experimental planning results.

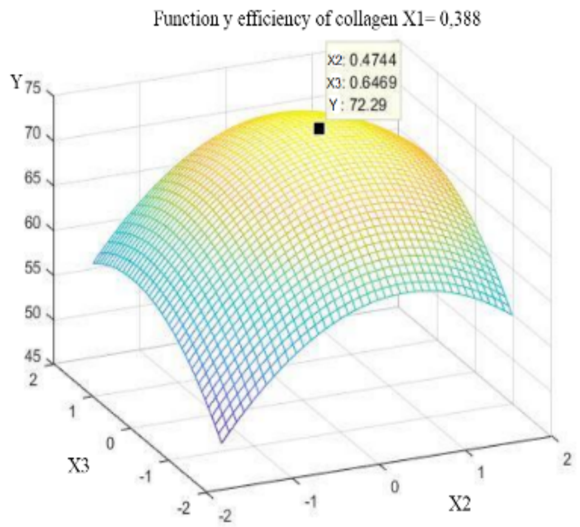

Figure 1. *Surfaces meeting collagen extraction performance*

3.4 Determine some properties of the finished collagen

The chemical compositions of the finished collagen were determined as in Table 5.

Table 5. *Chemical compositions of the finished collagen*

No.	Criteria	Unit	Content	Measurement methods
1	Moisture	%	8.54 ± 0.05	Dry to constant mass
2	Protein	%	82.49 ± 0.49	Kjeldahl
3	Fat	%	0.29 ± 0.02	Soxhlet
4	Mineral	%	0.21 ± 0.01	Incinerate
5	pH	-	6.55 ± 0.05	pH meter

The finished collagen is obtained by SEM and scanned at fibrous magnification of 50,000 times.

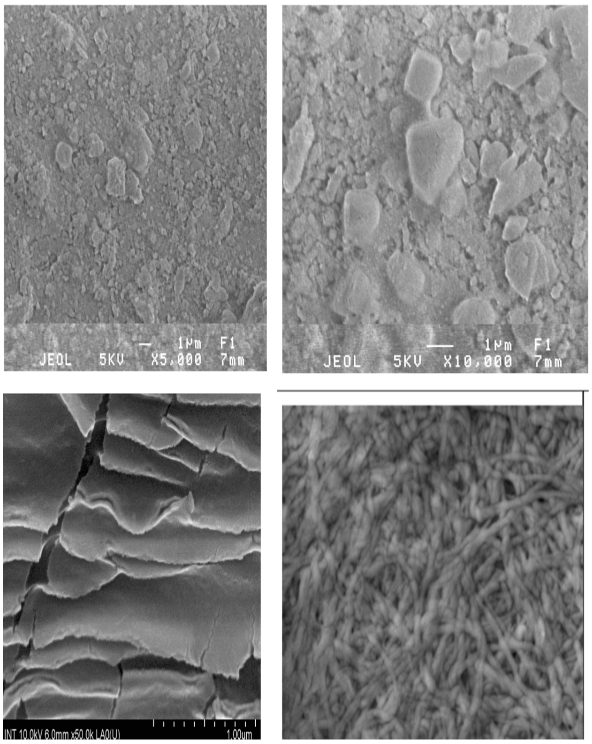

Figure 2. *Photo of the finished SEM collagen*

High levels of the amino acids Glycine, Arginine and Proline in collagen finished products are high, corresponding to the amino acid composition of collagen from ray skin in previous studies.

Table 6. *Amino acid compositions of the finished collagen*

No.	Amino acids	Ingredients (g/100g)
1	Alanine	6.49
2	Arginine	5.71
3	Aspatic acid	2.83
4	Glutamic acid	5.62
5	Glycine	9.74
6	Histidine	0.79
7	Isoleucine	1.20
8	Leucine	2.34
9	Lysine	2.04
10	Methionine	0.70
11	Phenylalanine	1.17
12	Proline	7.77
13	Serine	2.17
14	Threonine	1.50
15	Tyrosine	0.43
16	Valine	2.01

The result of collagen electrophoresis from ray skin was shown in Figure 3.

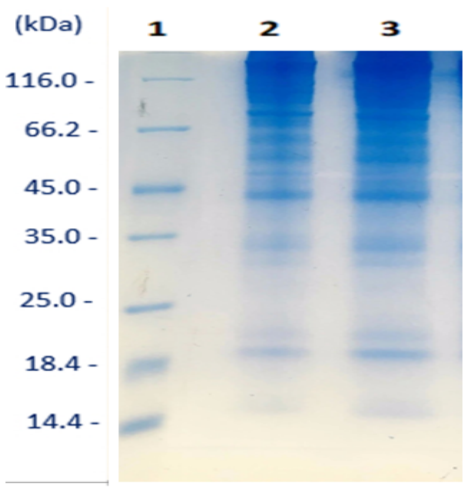

Figure 3. *SDS-PAGE collagen from ray skin 2- Marker, 2,3 - collagen from ray skin*

The above results showed that collagen from ray skin had a low molecular weight number of molecules because collagen is partially hydrolyzed.

The results of FTIR spectroscopy also showed that the results of the finished collagen were partially denatured.

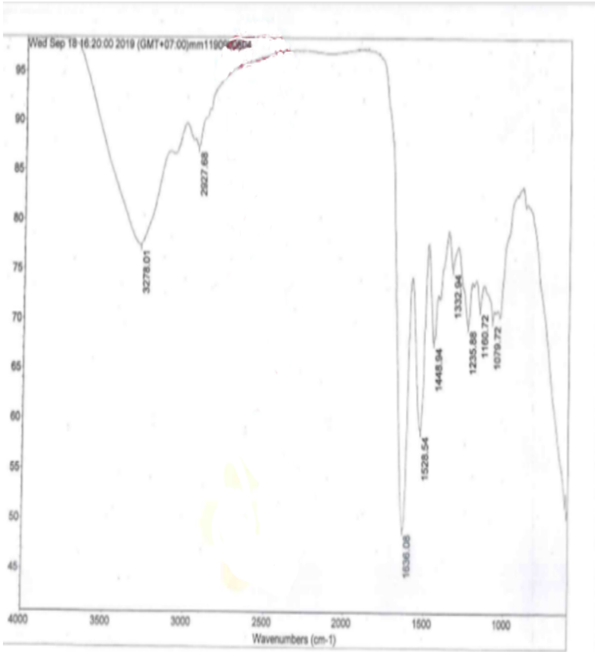

Figure 4. *FTIR spectrum of finished collagen product*

4. CONCLUSIONS

Collagen extraction process from ray skin was firstly by purified treatment steps with NaOH 0.075M at the rate of 1/10 (v/w) in 24 hours to remove non-protein compounds, treated with 1% H_2O_2, the rate of 1/10 (v/w) in 2 hours to remove color. The purified fish skin was extracted with 0.578M acetic acid at the rate of 1/11 (v/w) for 53 hours, then precipitated with NaCl salt solution with the highest collagen extraction efficiency which was 72.29 %. The precipitate was centrifuged at 3000 rpm for 40 minutes and refrigerated at 30°C for 24 hours to obtain high quality collagen products including protein of 82.49%, lipid of 0.29%, mineral of 0.21%, moisture of 8.54% and full of essential amino acids.

Optimization of the process showed that the mathematical model demonstrated the process of extracting collagen from stingray skin with maximum extraction efficiency of 72.29%. This result is the scientific basis for selecting the optimal conditions for collagen processing from the stingray skin byproducts with the highest quality, creating collagen with white color, soft fiber structure to meet the commercial collagen quality.

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