Optimization of Conditions for Extraction of Collagen from Pig Hide by Response Surface Methodology

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Abstract: Hide offal as a major part of tannery waste contains over 80% collagen. Collagen is a kind of excellent functional biomaterial as well as a good source of amino acids. Because of its special biological properties, collagen has good application foreground in medicine, food, cosmetic and agriculture. The process of extracting collagen by trypsin using the limed hide offal of pig hide as raw materials was presented. The optimal conditions for collagen extraction were determined by response surface methodology in this work. Box–Behnken design was applied to evaluate the effects of four independent variables (enzyme dosage, temperature, time and pH) on the collagen yield. Correlation analysis of the mathematical-regression model indicated that quadratic polynomial model could be employed to optimize the extraction of collagen. From response surface plots, enzyme dosage, temperature, time and pH exhibited interactive effects on the extraction of collagen. The optimal conditions to obtain the highest collagen yield of pig hide were an enzyme dosage at 403.8 U/g, a temperature at 50℃, a time at 1.9 h. and a pH at 7.5. Under these optimal conditions, the experimental values agreed with the predicted values, using analysis of variance, indicating a high goodness of fit of the model used and the success of response surface methodology for modeling extraction of collagen from pig hide.

Key words: collagen; enzymatic hydrolysis; response surface methodology

1 Introduction

Pig hide offal as a major part of tannery waste contains over 80% collagen. Leather mass loss in leather processing is up to 50%. The best way for their removal is to recover soluble collagen that may have commercial use. A number of authors have reported about chemical and enzymatic treatment of leather waste. [1, 2]

Treatment of the offal from fleshing machines has traditionally been limited to the chemical process of rendering. This is time consuming and necessitates the use of high amounts of energy. Exposure of the fat to high temperatures and strongly acidic or basic conditions will obviously yield products which will not give the most economical return. An alternative method is enzymatic processing, which is becoming increasingly more economical with the advent of biotechnology. Several studies on the enzymatic hydrolysis of various collagen sources such as fish scale, horn, hoof, leather waste, and animal bone have been reported. [3, 4] In Comparison with the liming and acid processes, the enzymatic process has several advantages such as short processing time and much less waste generated. These treatments can be carried out at low temperature, over short periods of time, yielding a protein product which can be used as a fertilizer or be disposed of by the sanitation departments.

Semrl-Kosmac reported that high amounts of collagens exist in pig hide offal. [5] Therefore, taking pig hide offal as a material to extract collagen is an effective attempt for remove the leather waste. Enzyme dosage, enzymatic temperature, time and pH are important variables affecting the extractability of collagen from pig hide offal. Response surface methodology is a statistical method that uses quantitative data from an appropriate experimental design to determine or simultaneously solve multivariate equation [6].

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Besides, this experimental methodology can generate a mathematical model and optimize the process levels[7, 8]. The objective of this work was to investigate the effects of above four variables on the yield of collagen extracted from pig hide offal by response surface methodology. Optimization of the extraction was also performed.

2 Materials and methods

2.1 Raw materials

Pig hide offal was purchased from Xi'an leather Industrial Company, China. The hide offal was denuded, descaled and washed with tap water. The cleaned offal were minced into small pieces (less than 4mm) by a cutting mill (ZB20L, Ruiheng Food Machine Co, Zhucheng, China) and frozen at −18 °C prior to collagen extraction.

2.2 Enzyme activity assay

The test method was modified from the assay of endo-protease using azo-casein. Enzyme activity was determined as the release of trichloroacetic acid (TCA) soluble after 20 min incubation with azo-casein substrate (0.2%, w/v). Blank tests were prepared by adding the TCA to the substrate solution immediately before enzyme was added. The solution was centrifuged at 3000 rpm for 20 min and the liquid phase was then taken to determine the absorbance of the spectrum at the wavelength of 440 nm. [2]

2.3 Pretreatment

Removal of inorganic compounds and salt-soluble collagen from pig hide offal was carried out by soaking in a 10% HCl solution at room temperature for 24 h. The concentration of pig hide offal in the solution was 10% (w/v). The procedure for removal of inorganic compounds and salt-soluble collagen was performed twice and washed with tap water. Then the defatted sample was soaked in 10% Na_2CO_3 solution at 40℃ for 2h with intermittent agitation for two times.. After removal of fat from pig hide offal, the sample was washed with distilled water and frozen at −18 °C prepared for collagen extraction.

2.4 Extraction of collagen by enzymatic hydrolysis

The defatted hide was subjected to collagen extraction by enzymatic hydrolysis. Enzyme dosage, enzymatic temperature, time and pH were chosen as variables with different levels. After enzymatic hydrolysis, the collected samples were rapidly heated to 90°C for 15 min to deactivate the enzyme. The sample was centrifuged for 15 min before filtration.

2.5 Box–Behnken design

Tab. 1 Experimental range and values for the independent variables

Independent variables	Symbols	Range and levels		
		-1	0	1
enzyme dosage（u/g）	X_1	200	400	600
time （℃）	X_2	6	7.5	9
pH	X_3	30	50	70
temperature（h）	X_4	1	2	3

Response surface methodology was employed for experimental design, data analysis and model building with software Design Expert (Trial Version 7.1.1, Stat-Ease Inc., Minneapolis, Minnesota, USA). A Box–Behnken design with three variables was used to determine the response pattern and then to establish a model[9, 10]. The enzyme dosage (X_1), enzymatic time temperature (X_2), pH (X_3) and temperature

(X_4) were chosen for independent variables. The range and center point values for the four independent variables were based on the results of preliminary experiments (Tab. 1). Yield of collagen was selected as the dependent variables for the combination of the independent variables were given in Tab. 2. Experimental runs were randomized in order to minimize the effects of unexpected variability in the observed responses.

Tab. 2 Central composite design and responses for the dependent variables

Run no.	Coded levels of variable				Response
	X_1	X_2	X_3	X_4	Y
1	-1	-1	0	0	56.69
2	-1	0	1	0	53.73
3	-1	0	-1	0	54.83
4	-1	0	0	-1	39.24
5	-1	0	0	1	32.98
6	-1	1	0	0	54.83
7	0	1	1	0	32.43
8	0	-1	1	0	35.4
9	0	0	-1	-1	47.58
10	0	0	-1	1	51.32
11	0	0	1	-1	44.51
12	0	-1	0	1	55.93
13	0	1	0	-1	55.16
14	0	1	-1	0	22
15	0	1	0	-1	55.6
16	0	-1	-1	0	42.2
17	0	1	0	1	55.49
18	0	0	1	1	46.26
19	1	0	1	0	52.74
20	1	0	-1	0	54.28
21	1	0	0	-1	24.64
22	1	0	0	1	58.78
23	1	1	0	0	57.68
24	1	-1	0	0	56.04
25	0	0	0	0	65.04
26	0	0	0	0	64.49

2.6 Analysis of data

The response surface regression (RSREG) procedure of Design Expert was used to fit the following quadratic polynomial equation. [11]

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \qquad (1)$$

Where Y is the dependent variable (yield of collagen, %), β_0 is a constant, β_i, β_{ii}, β_{ij} are regression coefficients and X_i, X_j are levels for the independent variables. Ridge Max option of RSREG procedure was used to compute the estimated ridge of optimum response for increasing radii from the center of the origin design. Multiple response optimizations were heuristically calculated in order to search the condition simultaneously satisfying two dependent variables (X_1 and X_2). The response surface plots were developed represented a function of two independent variables while keeping the other two independent variables at the optimal values.

3 Results and discussion

3.1 Diagnostic checking of the fitted model and data analysis

All 26 experimental points were evaluated and the results of the dependent variable (Y) for each point are shown in Tab. 2. All the coefficients of linear (X_1; X_2; X_3; X_4), quadratic (X_{11}; X_{22}; X_{33}; X_{44}) and interaction were calculated for significance with t-statistic and the estimated coefficients of each model are presented in Tab. 3. Among the quadratic coefficients, X_2 X_3 (P < 0.0001) had highly significant effects, while all the coefficients in linear terms were significant at P<0.001. After removing the non-significant terms, the response surface model equation shown below was established as follows.

$$\boldsymbol{Y = 64.76 + 1.36 X_1 - 1.37 X_2 + 1.50 X_3 + 0.48 X_4 - 10.02 X_2 X_3 - 8.02 X_1^2 - 10.99 X_2^2 - 17.59 X_3^2 + 3.11 X_4^2}$$

Where Y is the yield of collagen, X_1, X_2, X_3 and X_4 are the coded variables for enzyme dosage, time, pH and temperature, respectively.

Tab. 3 Estimated coefficients of the fitted quadratic polynomial equation for different responses based on *t*-statistic

Source	Degrees of freedom	Sum of Squares	Mean Squares	F-value	Pr>F
X_1	1	1232.3000	1232.3000	45.9683	< 0.0001
X_2	1	2104.3000	2104.3000	67.9683	< 0.0001
X_2	1	1527.0160	1527.0160	59.1730	< 0.0001
X_2	1	1032.7161	1032.7161	37.1179	0.0002
X_1 X_2	1	134.2017	134.2017	4.0131	0.0154
X_1 X_3	1	43.0370	43.0370	1.0884	0.2035
X_1 X_4	1	65.224	65.224	1.8285	0.1071
X_2 X_3	1	1145.3013	1145.3013	47.0131	< 0.0001
X_2 X_4	1	76.0000	76.0000	1.9075	0.0465
X_3 X_4	1	34.3646	34.3646	0.0158	1.9021
X_1^2	1	480.9026	480.9026	18.1969	0.0005
X_2^2	1	526.8103	526.8103	22.8743	0.0006
X_3^2	1	1349.9349	1349.9349	58.6148	< 0.0001
X_4^2	1	431.1477	431.1477	15.0064	0.0007

In general, exploration and optimization of a fitted response surface may produce poor or misleading results unless the model exhibits a good fit, which makes checking of the model adequacy essential. [12] The P-value of the model was 0.0002 (Tab. 3). Meanwhile, the lack of fit value of the model was 0.84 which was not significant. These two values confirmed that the model fitness was good.

Tab. 4 Analysis of variances for the response surface quadratic model of the yield of collagen

Source	Degrees of freedom	Sum of Squares	Mean Squares	F-value	Pr>F
Model	14	2785.7089	798.9792	29.6398	0.0002
Residual	11	553.3370	23.0306		
Lack of Fit	10	253.1872	21.3187	9.17	0.84
Pure error	2	0.1498	0.1498	0.25	
Cor Total	25	12883.9511			

Coefficient of determination (R^2) is defined to be the ratio of the explained variation to the total variation and is a measurement of the degree of fitness. [13] A small value of R^2 indicates a poor relevance of the dependent variables in the model. The model can fit well with the actual data when R^2 approaches unity. By analysis of variance, the R^2 value of this model was determined to be 0.9714, which proved that the regression model defined well the true behavior of the system.

3.2 Conditions for optimum responses

To optimize collagen extraction, the central points were decided by central composite design[7]. The design had four independent variables for the enzyme dosage (X_1), time (X_2), pH (X_3) and temperature (X_4); their central points and ranges were determined by preliminary data (Tab. 1). The coded and uncoded values in the optimum condition obtained from the results of RSM are shown in Tab. 5.

The results of the RSREG procedure revealed that all the eigenvalues were negative, so the maximum values for collagen yield (Y, %) are shown in the stationary point of Fig. 1. The coded values for the optimum collagen extraction from pig hide off were 0.0013 for enzyme dosage (X_1), -0.0257 for time (X_2), 0.01435for pH (X_3) and 0.0037 for temperature (X_4). Their uncoded values were 403.8 u/g enzyme dosage (X_1), 1.889h time (X_2), pH 7.513 (X_3) and 50.322 ℃ temperature (X_4) (Tab. 5). The predicated content of the dependent variable (Y, contents) was 69.7% from calculations using the model equation derived from the above optimal conditions. The experimental value for collagen content using the optimum conditions was 68.3%, which was slightly higher, 1.4%, but in close agreement with the predicted value.

Tab. 5 Optimal conditions for processing collagen from pig hide offal

Dependent variables	Independent variables	Critical value		Predicted value	Experimental value
		Coded	Uncoded		
Y (yield, %)	X_1	0.0013	403.8 u/g	69.7%	68.3%
	X_2	-0.0257	1.889h		
	X_3	0.01435	7.513		
	X_4	0.0037	50.322 ℃		

3.3 Effects of the enzyme dosage, temperature, pH and time on the yield of collagen

Two three-dimensional graphs of each dependent variable(Y) plotted against two independent variables (X_i, X_j) were drawn with Expert design and are shown in Fig. 1-4.

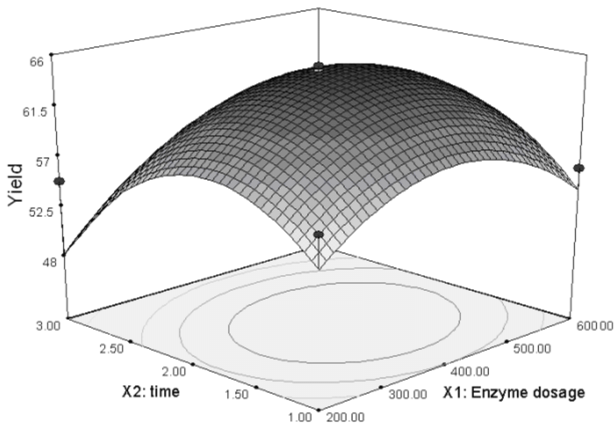

Fig. 1 Response surface plot showing the effects of enzyme dosage and time on the yield of collagen. The temperature and pH was constant at 30.0 °C and 7.5

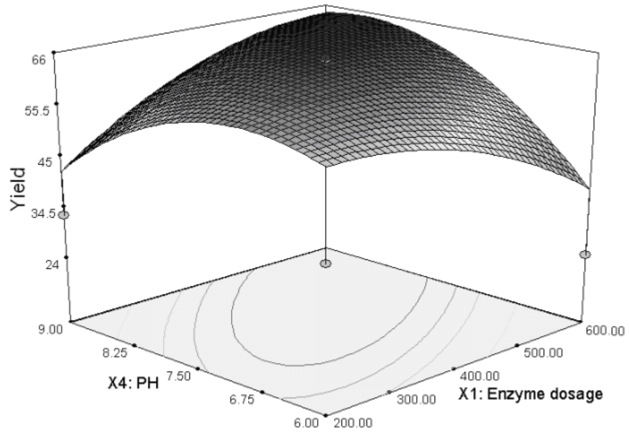

Fig. 2 Response surface plot showing the effects of enzyme dosage and enzymatic pH on the yield of collagen. The enzymatic temperature and time was constant at 30.0 °C and 2h

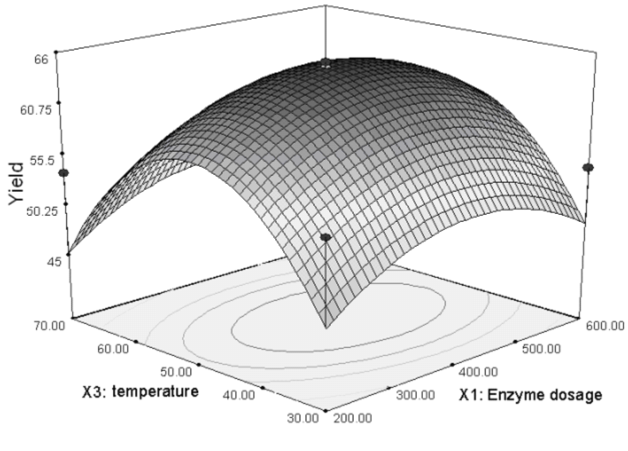

Fig. 3 Response surface plot showing the effects of enzyme dosage and temperature on the yield of collagen. The enzymatic time and pH were constant at 2h and 30.0 °C

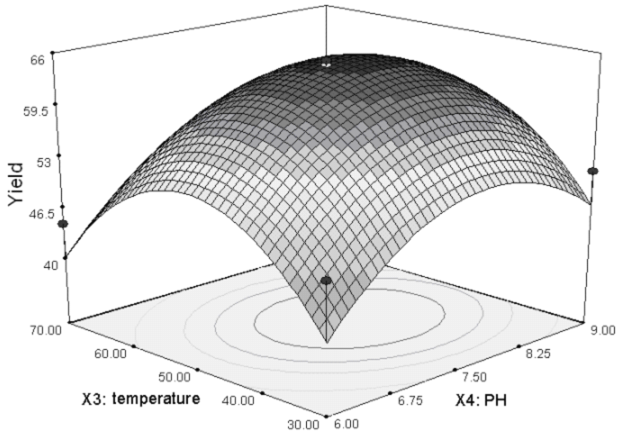

Fig. 4 Response surface plot showing the effects of enzymatic time and temperature on the yield of collagen. The enzyme dosage and pH were constant at 400u/g and 30.0 °C

The effects of enzyme dosage and enzymatic time on the yield of collagen from pig hide offal are shown in Fig. 1.The yield of collagen increased with the extension of time, especially when the time was within the range of 1–2 h. thereafter a further decrease was shown when the time was longer. This may be due to that part of the collagen may be hydrolyzed and dissolved in the solution with the prolonged enzymatic time.

From Fig. 2, a positive relation was found between enzyme dosage and the yield of collagen at suitable enzymatic pH. The yield improved with the increase of enzyme dosage. But lower or higher of enzymatic pH led to an inhibition of enzyme activity, which led to a decreased yield.

Fig. 3 and Fig. 4 depicted the effects of independent variables of enzymatic temperature, time and enzyme dosage on the yield. A similar effect was observed to that for the enzymatic time. There was an optimal value for temperature to obtain the highest yield of enzymatic temperature. Collagen is a thermo-instable protein which can be denatured at room temperature. This sensitivity to temperature is associated with its chemical structure. This is due to the presence of hydroxyproline in inter-chain linkage by hydrogen bonds, which stabilizes the triple-helix structure of collagen[14]. Therefore, control of temperature during the extraction of collagen is important to keep the natural structure of collagen. Increase of temperature to a certain extent can facilitate the decomposition of collagen into gelatin which increases the yield of pig hide off extraction. However, relevant high temperature can only change the conformation of collagen which might decrease the solubility. This might explain the decrease of yield of collagen when 70 °C was applied in this work.

4 Conclusions

From the results obtained, the four variables (enzyme dosage, enzymatic temperature, time and pH) had significant effects in the extraction of acid-soluble collagen. The collagen yield increased with the rise of enzymatic temperature, time and pH to a certain value, thereafter decreased. A positive relation was found between enzyme dosage and yield of collagen. The mathematical model gave an R^2 of 0.9714 and a P-value of less than 0.0002, which implied a good agreement between the predicted values and the actual values of the yield of acid-soluble collagen, and confirmed a good generalization of the mathematical model. The optimal conditions to obtain collagen were determined as follows: a 403.8 u/g enzyme dosage, 1.889h time, pH 7.513 and 50.322℃ temperature. The predicted yield of collagen was 69.7% which was in agreement with the actual value 68.3%. The results in this work were helpful for the production of collagen from pig hide offal. However, further research on the structural characteristics of collagen is important to understand its physical and chemical properties.

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