

Application of Response Surface Methodology to Optimize Hydrolysis of Collagen from Croaker Scale

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Abstract: Response Surface Methodology (RSM) based on a Central Composite Rotatable Design (CCRD) was applied to optimize the hydrolysis of collagen from croaker scale with the aim to maximize the Degree of Hydrolysis (DH). Effects of pH, temperature, the ratio of Enzyme to Substrate (E/S) and hydrolyzing time on DH were investigated. The results showed that the DH was predicted very well by a second-order polynomial model ($R^2 = 0.9153$, $p < 0.0001$). The highest DH was theoretically predicted to be 45.43%, under the optimal conditions (pH 8.0, 54.0 of temperature, 3.0% of E/S and 2.3 h of hydrolysis time). The recovery experimental DH under optimal conditions was calculated to be 46.63%, which confirmed the reliability of the method.

Keywords: Central composite rotatable design, degree of hydrolysis, response surface methodology, scale collagen

INTRODUCTION

Collagen is a main constitution protein of skin, bone, tendon and other kinds of connective tissue (Foegeding *et al.*, 1996). It is researched for its biological compatibility, biological degradability, gel-forming properties and so on (Li *et al.*, 2004; Gómez *et al.*, 2011), what can be widely applied in food, pharmaceutical and cosmetic industries (Cavallaro *et al.*, 1994; Hassan and Sherief, 1994). In food industry, because of its bioactives and nutrition value (Montero *et al.*, 1995), collagen is often used as ingredients and additives of functional food and nutraceuticals. While collagen is hard to absorb through oral intake for its high molecule weight. One way to solve this problem is to hydrolyze the collagen with appropriate protease. Enzymatic hydrolyzing process can produce small fragments of collagen peptides. Furthermore, some of its bioactive increased obviously (Huo and Zhao, 2009) and its antigenicity decreased (Fujita and Yoshikawa, 1999) after hydrolysis. Therefore, it is an ideal method to produce functional food enriched collagen peptides through enzymatic hydrolysis. In order to produce the bioactive peptides, quantities of collagen resources are required. The skin and bones of mammals, such as pig and cattle, were good resources of collagen (Jongjareonrak *et al.*, 2005). But now it is queried for the threaten of carrying bovine spongiform encephalopathy (BSE) or Foot and Mouth Disease Virus (FMDV) (Sadowska *et al.*, 2003; Helcke, 2000). Some new collagen resources including fish skin and scale were obtained more and more attention (Karim and Rajeev, 2009). And it is also a process of appreciation to utilize these food industrial by-products. Besides, extensive researches have reported that the collagen peptides derived from variety of fish have

various bioactives (Fahmi *et al.*, 2004; Mendis *et al.*, 2005; Jung *et al.*, 2006; Huo *et al.*, 2009; Gómez *et al.*, 2010; Ngo *et al.*, 2010). While as we could know, the collagen of large yellow croaker (*Pseudosciaena crocea*) scale has not been studied so far. The aim of this study was to optimize the hydrolysis conditions of croaker scale collagen by optimal protease which was chosen through former studies (unpublished) with Response Surface Methodology (RSM) based on a Central Composite Rotatable Design (CCRD). To reduce the time and save the cost, central composite rotatable design is widely used in experiment to replace conventional design of studying one factor at a time. Response surface methodology has been a quite effective method of statistical and mathematical analysis for experiment data since it was first proposed by Box and Wilson (1951). It can evaluate the influence of all the variables in the multiple factor experiment design. What's more, the mutual interaction among factors can also be estimated simultaneously (Myers *et al.*, 2008). And a polynomial regression equation can be given to predict the optimal condition of factors on the response. Here, we employed four main factors (pH, temperature, the ratio of enzyme to substrate (E/S), time) as variables and five levels to optimize the hydrolysis of fish scale. The Degree of Hydrolysis (DH) was set as response to evaluate the efficiency of hydrolysis.

MATERIALS AND METHODS

Materials: Croaker scale were provided by Yingzhou Seafood Corporation. (Zhoushan city, Zhejiang province, China). It was dried, packed in a polyethylene bag and stored in a cool and dark place until used. Alcalase (200 U/mg) was purchased from Shanghai

Table 1: Uncoded and coded levels of independent variables used in the RSM design

Factors	Coded variable	Levels				
		-2	-1	0	+1	+2
pH	X ₁	7.0	7.5	8.0	8.5	9.0
Temp	X ₂	45	50	55	60	65
E/S	X ₃	1.5	2.0	2.5	3.0	3.5
Time	X ₄	1.0	1.5	2.0	2.5	3.0

Kayon Biological Technology Co., Ltd. (Shanghai, China). Trinitrobenzene sulfonic acid (TNBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other reagents were from commercial resource and of analytical grade.

Pretreatment of croaker scale: The scale was soaked in 0.4 M HCl for 6 h to remove the calcium and washed with ultrapure water to eliminate the acid used. Then we soaked the scale in 0.1 M NaOH for 12 h to remove impure protein and washed the scale to eliminate alkali as previously described. The scale was dried at 50°C for 24 h and minced, ready for use.

Hydrolysis of scale collagen: 0.2 g scale sample and 30 mL 0.1% (v/v) HAc were put into a 50 mL conical flask. The flask was sealed and placed in autoclave to be treated under 121 1.1 Mpa for 1h. Afterwards the flask with the mixture was natural cooled and the pH was adjusted to a defined value (ranging from 7.0 to 9.0) with 10 M NaOH. Different quantities (E/S (enzyme/collagen) ranging from 1.5 to 3.5%) of alcalase was added into the system to start hydrolyzing at a given temperature (ranged from 45 to 65) and was incubated for a certain time(0.5 h, 1.0 h, 1.5 h, 2.0 h, 2.5 h, 3.0 h). Then it was deactivated in boiling water bath to terminate the reaction.

Determination of the Degree of Hydrolysis (DH): The DH of the hydrolyzate was calculated according to TNBS method (Nissen- Adler, 1979) with some modifications. 1.0 mL of the sample was mixed with 2.0 ml of phosphate buffer (pH 8.2) and 1.0 mL of 0.1% TNBS. The mixture was incubated for 30 min in the dark and the reaction was terminated by the addition of 2.0 mL of 0.1 M Na₂SO₃. The absorbance was then measured at 420 nm after the mixture was kept for 15 min. the DH was evaluated as Eq. (1):

$$DH(\%) = (N_1 - N_3) / (N_2 - N_3) \times 100 \quad (1)$$

where,

- N₁ = Free amino nitrogen of hydrolyzate, mmol/g
- N₂ = Total amino nitrogen of the scale collagen, mmol/g
- N₃ = Free amino nitrogen of the scale collagen, mmol/g

Total amino nitrogen of scale collagen was measured after digestion by 3M H₂SO₄.

Experimental design: Previous single factor experimental data was used to be the guiding parameters for establishing the range of the factors in the experiments. Response Surface Methodology (RSM) based on Central Composite Rotatable Design (CCRD) was applied to evaluate the effects of four controlled independent variables, pH (X₁), temperature (X₂), E/S (X₃), time (X₄) and their interaction on the measured response, DH (Y). The independent variables were coded at five levels (-2, -1, 0, +1, +2) and the complete design consisted of 31 experimental points including 7 replications of the centre points. The coded levels of the independent variables used in the RSM design are listed in Table 1.

Statistical analysis: The second-order polynomial model proposed for the response surface analysis of the designed experiment was explained by Eq. (2):

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

where, Y is the DH; β_0 , β_i , β_{ii} and β_{ij} are the coefficients of intercept, linear, quadratic and interactive terms respectively; while X_i and X_j are the coded values of the four independent variables.

To analyze the multiple regression and variance, develop a regression equation between variables and response and numerical optimize the procedure, the SAS software program (version 9.0, SAS Institute Inc., Cary, NC, USA) was employed.

RESULTS AND DISCUSSION

Single factor experiments: The process of the hydrolysis had been described before. Three of four factors were fixed (pH 8.0, temperature 50, E/S 2.5%, time 3.0 h) while the other factor was ranged to evaluate its effect on hydrolyzing. The DH of the hydrolyzate could be measured by TNBS method and the results were shown in Fig. 1a to d respectively.

It can be seen from Fig. 1a that the DH increased with increasing pH and reached a maximum at the pH of 8.0, while decreased gradually with further increase of pH. Therefore, the pH of 8.0 was adequate for the function of alcalase in the process of hydrolysis.

As indicated in Fig. 1b, the DH started to increase with the increase of temperature from 45 and reached a

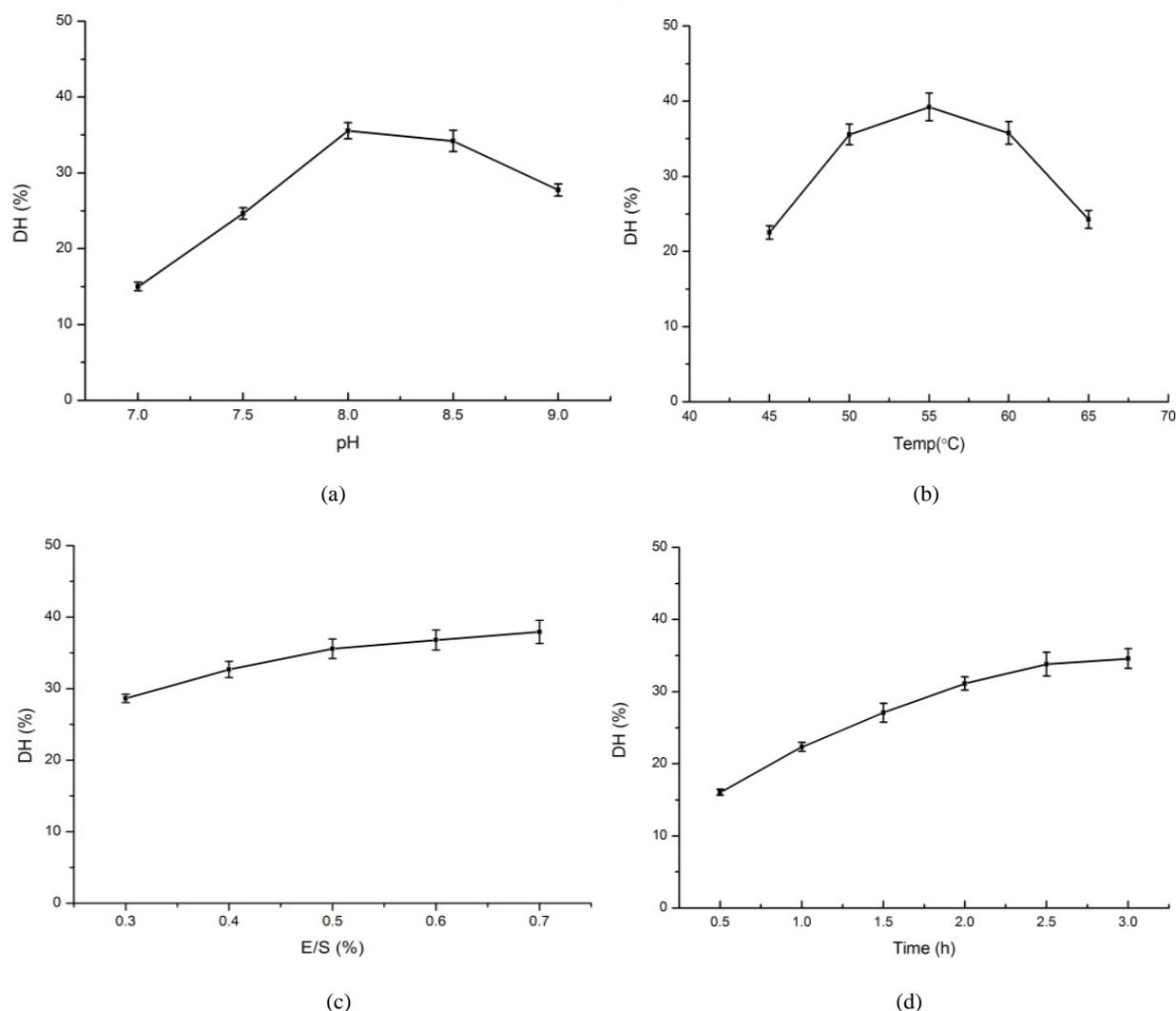


Fig. 1: a, b, c, d: the effects of pH (a), temperature (b), E/S (c) and time (d) on the degree of hydrolysis (DH). The value was labelled as average value of three replicates and the error bar was labelled as standard errors

peak at 55, then decreased significantly. An appropriate incubation temperature could be conducive to the function of the enzyme and increase the reaction rate, while excessive temperature would also decrease the activity of enzyme. In this single factor experiment, 55 could be chosen as an optimum incubation temperature.

As can be seen from Fig. 1c, there was an increase in the DH with the increase of E/S, while the tendency of the increase diminished gradually. Despite of the aim to maximise the DH, the cost and the economy should be taken into consideration as well. It is a waste to pursue higher DH with excessive enzyme. Thus, 2.5% of E/S was adequate.

It can be seen from Fig. 1d that the tendency of the DH increase with increasing incubation time was similar as that in Fig. 1c. Too long time means too much expenditure of energy and so on. For the same reasons, time of hydrolyzing should be controlled at 2.0 h.

Response surface methodology experiments:

According to the results obtained in single factor experiments, RSM was employed to optimise the condition of hydrolyzing process. The experimental data was given in Table 2. Multiple regression analysis of the data gave the second-order polynomial regression equation between variables and response:

$$Y = 43.30 + 0.35X_1 + 1.51X_2 + 2.90X_3 + 3.23X_4 - 2.75X_1^2 - 2.73X_2^2 - 2.02X_3^2 - 3.78X_4^2 - 0.47X_1X_2 - 0.24X_1X_3 + 0.20X_1X_4 - 2.77X_2X_3 + 0.21X_2X_4 + 0.88X_3X_4$$

ANOVA was used to evaluate the significance and suitability of the response surface model. The statistical data of the test was shown in Table 3. As it manifested, the model for the hydrolysis was highly significant ($F = 12.35$, $p < 0.0001$), so there was only a 0.01% chance that a model F value this large could occur due to noise.

Table 2: Factors and levels in the RSM based on CCRD and experimental results

No.	X ₁	X ₂	X ₃	X ₄	DH (%)
1	+2	0	0	0	36.30
2	+1	+1	-1	-1	29.06
3	+1	-1	-1	+1	26.46
4	-1	-1	-1	+1	22.93
5	-1	+1	-1	-1	28.28
6	0	0	0	0	44.07
7	+1	+1	+1	-1	28.09
8	0	-2	0	0	32.90
9	0	0	0	0	43.61
10	-1	+1	+1	+1	37.88
11	0	0	+2	0	42.1
12	0	0	0	0	43.14
13	-1	+1	-1	+1	34.22
14	-1	-1	+1	-1	29.99
15	0	0	0	-2	25.05
16	-1	-1	-1	-1	21.12
17	0	0	0	0	44.07
18	0	0	0	0	41.99
19	+1	-1	-1	-1	20.36
20	0	0	0	+2	37.27
21	-2	0	0	0	34.27
22	+1	+1	+1	+1	36.01
23	0	0	-2	0	34.28
24	+1	-1	+1	-1	31.57
25	-1	+1	+1	-1	29.14
26	0	+2	0	0	37.80
27	0	0	0	0	43.41
28	+1	+1	-1	+1	34.75
29	+1	-1	+1	+1	40.06
30	-1	-1	+1	+1	38.46
31	0	0	0	0	42.80

The coefficient of determination (R^2) and adjusted coefficient of determination (Adj. R^2) of the polynomial model were 0.9153 and 0.8412 (Table 3). Thereby the R^2 of 0.9153 was acceptable for the acceptance of $R^2 > 0.75$ that had confirmed by Chauhan and Gupta (2004). The linear terms E/S (X_3 , $p = 0.0001$) and time (X_4 , $p < 0.0001$) were highly significant, temperature (X_2 , $p = 0.0190$) was significant, while pH (X_1 , $p = 0.5542$) was not significant. The quadratic terms X_1^2 ($p < 0.0001$), X_2^2 ($p < 0.0001$), X_3^2 ($p = 0.0015$) and X_4^2 ($p < 0.0001$) were highly significant. All the interaction

terms had no significant influence on the response, except X_2X_3 ($p = 0.0012$) was highly significant. As a conclusion, the most effective variables were time and E/S of the hydrolyzing process, after what was the temperature significantly influencing the response DH.

Three-dimensional (3D) response surface plots presenting the effects of the four independent variables on the response were shown in Fig. 2. Each of the plots in Fig. 2 was drawn to illustrate two of the variables and their interaction affecting the dependent variable with another two variables fixed.

As shown in Fig. 2a, the increase of temperature affected the DH significantly when pH was at a certain value. However, when the temperature was at a certain value, the pH had no significant effect, neither did their interaction have a significant effect on DH. From Fig. 2b and c, it can be seen that the linear effects of E/S and time were significant on DH, but the mutual interactions between pH and any of the two factors were not so obvious. Figure 2d showed noticeable quadratic effect of interaction between temperature and E/S. As can be seen from Fig. 2e and f, the combined effects of temperature and time and the mutual interaction between E/S and time were not very obvious. These graphs met well with the results of ANOVA and described the statistical data visually. In each of the plots in Fig. 2, there was a peak of the curved surface that revealed an optimal condition of the factors on the response, which meant that any level of the condition lower or higher than that would produce a negative influence on hydrolyzing process with the result of diminishing the DH.

Optimisation and verification: To maximize the DH, the conditions of four factors in this study were optimized with SAS software and were recommended as: 8.03 of pH; 54.01 of temperature; 2.98% of E/S; and 2.27 h of time. Under these conditions, the response

Table 3: ANOVA for the second-order polynomial model

Source	SS	df	Mean square	F value	p-value
Model	1392.64	14	99.47	12.35	<0.0001*
X ₁	2.94	1	2.94	0.36	0.5542
X ₂	54.84	1	54.84	6.81	0.0190
X ₃	202.19	1	202.19	25.10	0.0001*
X ₄	250.91	1	250.91	31.15	<0.0001*
X ₁ X ₂	3.57	1	3.57	0.44	0.5150
X ₁ X ₃	0.91	1	0.91	0.11	0.7409
X ₁ X ₄	0.66	1	0.66	0.081	0.7790
X ₂ X ₃	123.21	1	123.21	15.29	0.0012*
X ₂ X ₄	0.73	1	0.73	0.091	0.7671
X ₃ X ₄	12.39	1	12.39	1.54	0.2328
X ₁ ²	216.07	1	216.07	26.82	<0.0001*
X ₂ ²	213.52	1	213.52	26.51	<0.0001*
X ₃ ²	116.98	1	116.98	14.52	0.0015*
X ₄ ²	408.60	1	408.60	50.72	<0.0001*
Residual	128.89	16	8.06		
Lack of fit	125.61	10	12.56	22.94	0.0005
Pure error	3.29	6	0.55		
Cor total	1521.53	30			

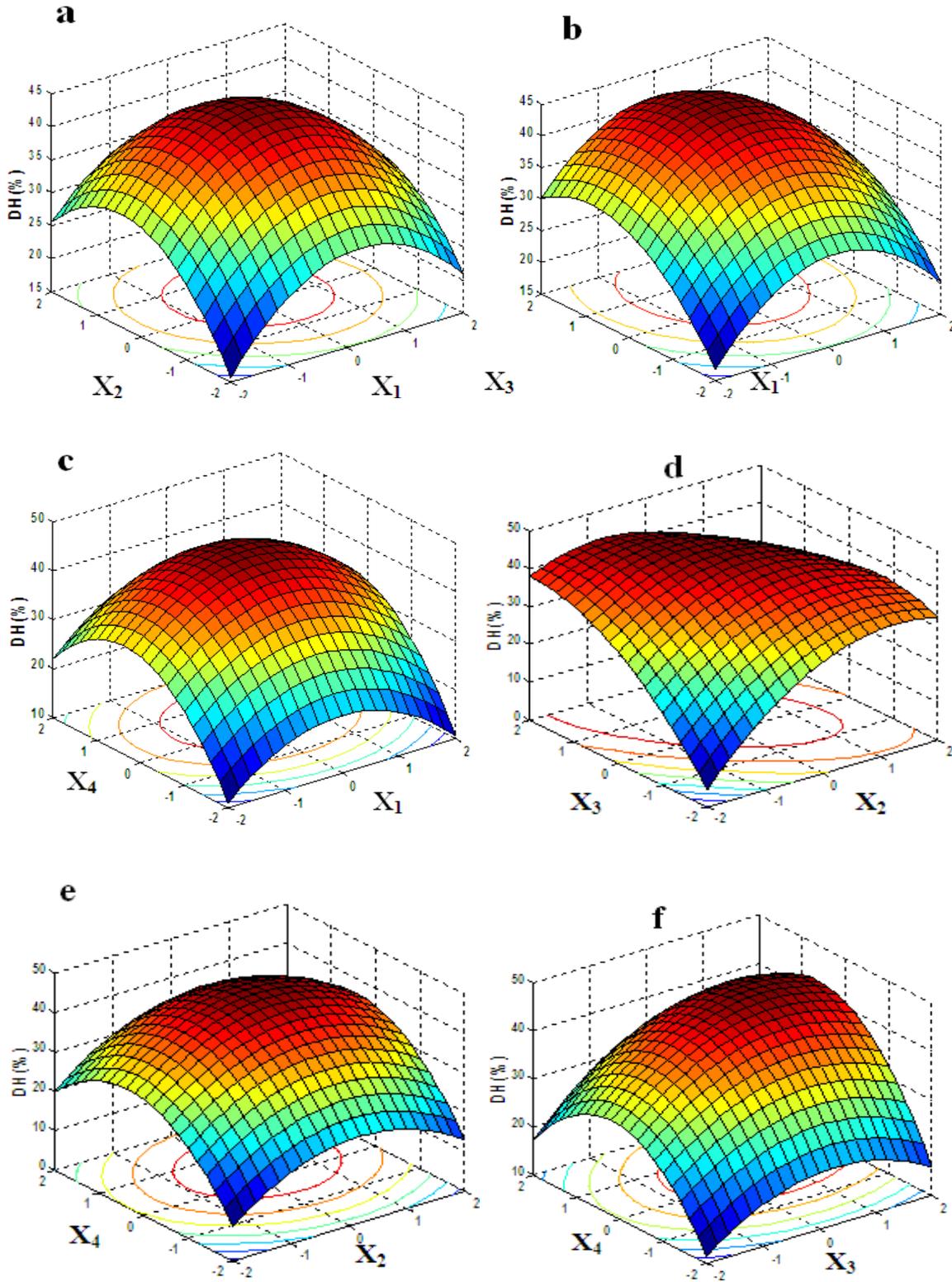


Fig. 2: Effects of different variables (X1: pH, X2: temperature, X3: E/S, X4: time) on the response (DH) presented in response surface (3D) plots

DH was estimated to be 45.43%. Three verification experiments were performed to evaluate the reliability of the model and the practical condition was controlled

to be: 8.0 of pH, 54.0 of temperature, 3.0% of E/S and 2.3 h of time. The average value of DH was observed as 46.63% and it showed only 2.65% of relative standard

deviation, which might be caused by the measurement and spectrophotometric method. Therefore the prediction of the provided model was reliable and could be applied to improve the hydrolysis of scale collagen.

CONCLUSION

This study used RSM and CCD to evaluate the effects of pH, temperature, E/S and time on the DH of large yellow croaker scale collagen with alcalase. The DH was dependent on hydrolyzing time, E/S, temperature and the mutual interaction between temperature and E/S. A second-order polynomial regression equation was given to describe the model and predicted an optimal condition of factors to maximize the DH. The predicted DH of 45.43% was very close to verification actual DH of 46.63% obtained under the optimal hydrolysis conditions of pH 8.0, temperature of 54.0°C, E/S of 3.0% and time of 2.3 h.

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