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Optimization of Extraction of Milkfish (*Chanos chanos, forskal*) Gelatin using Rsm-Bbd (Response Surface Methodology Box Behnkendesign)

[1]*Masirah, [2]Widjanarko, S. B. Dan, [3]Yuwono, S. S.

[1]Master Program of Food and Technology Science, University of Brawijaya, Indonesia
[2]Departement of Agricultural Product Technology, Faculty of Agricultural Technology, University of Brawijaya, Indonesia
3Departement of Agricultural Product Technology, Faculty of Agricultural Technology, University of Brawijaya, Indonesia

Abstract : Milkfish bone is one of the potential waste of fishery products as raw materials for halal gelatin. Conditions of the extraction process such as the extraction of HCL, its extraction time, and extraction temperature will affect the quantity and the quality of gelatin produced. Program of Design Expert 7.1.5 withResponse Surface Methodology Box-Behnken Design (RSM-BBD) is used to observe and choose the process condition from the combination of factor level which produces optimum response. RSM-BBDreported that concentration of HCl (X_1), pre-treatment time (X_2) and extraction temperature(X_3) are the highly influential factor on the value of yield response (Y_1) dan the gel strength (Y_2) of gelatin. Optimization results with RSM-BBD showed that the optimum conditions to process the extraction of milkfish bone gelatin obtained when concentration of HCl reached 4,65%, pre-treatment time was 26,89 hours and the extraction temperature reached 89,92 ^{0}C with yield result as much as 12,93% and the gel strength as much as 335,57 gram bloom. Verification results showed that the gelatin of fish bone extracted with optimum conditions had yield value as much as 12,97% and the gel strength as much as 335,57 gram bloom.
Keywords : Gelatin, Milkfish bone, RSM, Box-Behnken Design, Yield, Gel Strength

I. Introduction

Milkfish bone is one of the fish waste fishery products that have been getting neither the optimal attention nor handling. The attempt of the utilization of boneless milkfish is highly required not only for increasing economical values but also to prevent environmental polutions.Milkfish bone has potential as the sorce of collagen [1] and as the raw material for halal gelatin [2]. The gelatin from fish bone is the possible alternative for beef and pork gelatin. Commonly, the gelatin yield derived from fish bone is about 12% [3], so that it is estimated that from 910 ton of milkfish waste, it would be obtained 109,2 ton of gelatin. One of major advantage of fish gelatin source is that they are not related with the risk of mad cow disease. Fish bone gelatin is also accepted by Muslims, Kosher (Jewish) and serve as an alternative for markets concerned about Bovine Spongiform Encephalopathy (BSE) [4].

Fish gelatin derived from its bone and skin is grouped as gelatin type A which used acid process to initiate the extractiom process.The extraction with acid process is shorter than with alkaline process. Fish gelatin has lower melting and gelling temperature along the gel strength compared with mammals gelatin. The factors on the process of gelatin such as the concentration treatment, time treatment, and the temperature of extraction will affect gelatin's yield [5].Before the extraction, the method of preservation on raw materials also affect some physical characteristics of fish gelatin [6].

The main goal from gelatin extraction of milkfish bone is to obtain the possibility of maximum yield from hydroxyproline. Optimization is the method to find the best alternative from alternative set of certain experiment. Optimization is the experimental design wich is derived in modern statistic which is sought as the way to reach the goal with lowest price thoroughly [7].Response surface methodology has been effective in the optimization and monitoring of the food process [8].This method is a collection of mathematical and statistical modeling techniques that relate to the treatment of products to output and setting the regression equation to describe the relationship between the input parameters and properties of the product [9].

II. Materials and Methods

A. Materials

Milkfish bone used has a large dorsal spine specification with length of ± 18 cm, diameter ± 5 mm, ± 7 grams weight, derived from Unit Usaha Pengolahan Bandeng Tanpa Duri Politeknik Kelautandan Perikanan Sidoarjo. All chemicals used is using pro analysis (p.a) material except for certain materials. All of these chemicals are obtained from CV. MakmurSejati Malang.

B. Extraction Methods

Gelatin extraction step process begins with the degreasing on milkfishbones. Degreasing process is carried out to remove impurities, the rest of the meat and fat on the bones of fish. This process is done by soaking the bone in water with a temperature of 80 ^{0}C for 30 minutes. The next step, bones were washed, drained and dried. Bones weighed as much as 100 grams and then extracted with HCl concentration and the time of extraction based on the design of the experiment described in Table 2.Comparison of bone with an acid solvent (HCl) respectively is (1: 4), then it was performed the washing process back up to neutral pH (6-7). The process was followed by extraction with aquadest (1: 3) at a temperature of extraction based on the experimental design (Table 2) for 5 hours using a waterbath.Once it was done, the next is filtering and then drying the gelatin with an oven and the final product obtained was in the form of dry gelatin (gelatin granules).

C. Analysis Methods

1. Yield [10]

Gelatin yield observations was made by weighing the dry gelatin, then it is compared to the weight of fish skin (wet sample) multiplied by 100%. The amount of gelatin yield is calculated by the following formula:

$$\text{Yie}\boldsymbol{ld}\ (\%) = \frac{\text{The dry weight of gelatin}}{\text{Fresh bone weight}}\ \text{x 100 \%}$$

Notes :

A = dry weight of gelatin (gram)

B = weight of bone samples (gram)

2. Gel Strength [11]

Gel strength is measured in the following manner:

a. Gelatin is dissolved with aquadest with a temperature of 60 ^{0}C to obtain a concentration of gelatin solvent 6.67% (w / v).

b. The solvent was stirred using a magnetic stirrer until it became homogeneous, then it was poured in bloom standard jars (containers with a diameter of 3 cm and 2.7 cm high). Let it stand for 2 minutes.

c. Cooled in a refrigerator at a temperature of 10 ^{0}C for 16-18 hours to form a gel.

d. Gel strength was measured by means of Tensile Strength Instrument (Gause models Imada Digital Force / ZP-200 N), the load cell 5 kn and 1 mm diameter flat-surface teflon cylinder. Probe speed of 0.5 mm / s at a depth of 4 mm. Gel strength (maximum strength) is expressed in grams of force.

D. Experimental Design

Optimization of the extraction process of milkfish bonegelatin is done by using Response Surface Methodology Box Behnken Design (RSM-BBD) with a variation of 3 levels cconcentration of HCl (X_1,%), 3 level pre-treatment time (X_2, h) and 3 levels of extraction temperature (X_3, ^{0}C).Response parameters measured were yield (Y_1,h) and the gel strength (Y_2, gram bloom).The combination of three factors treatments were concentration of HCl (X_1,%), pre-treatment time (X_2, h) and extraction temperature(X_3, 0C), which is the independent variable and the yield (Y_1,%) and the gel strength (Y_2, g) which is a controlled variable (response).The center (code = 0) obtained from data on the concentration of HCl, pretreatment time and extraction temperature based on the results of the preliminary study and previous research data.The determining of minimum requirement (code = - 1) and maximum requirement (code = +1) of the independent variable were determined based on preliminary research.Optimization of the process of extracting gelatin with RSM is done to find the optimum level of concentration of HCl, pre-treatment time and extraction temperature needed by the extraction of milkfish bone gelatin.

After obtaining the optimum conditions the concentration of HCl, pretreatment time and extraction temperatur, then the verification was carried out. Verification formulations was carried out with milkfish bone gelatin extraction process at its optimum condition. The verification process is determined based on the equation model that has been built from optimization process using response surface methodology using Design Expert 7.1.5 program which includes analysis of model selection, analysis of variance (ANOVA) on the surface of response, response graph and determining the optimum point.The value of independent variables in this study can be seen in Table 1, whereas the experimental design to RSM-BBDresearch can be seen in Table 2 below.

Table 1. Independent Variables In BBD Research Design

Variable	**Level Code**		
	-1	**0**	**1**
Concentration of HCl, X_1 (%)	3	5	7
Pre-treatment time, X_2 (hour)	12	24	36
Extraction Temperature, X_3(^{0}C)	85	90	95

E. Statistical Analysis

Analysis of data for optimization of extraction conditions using RSM method with the help D.X Design Expert software version 7.1.5 (Stat-Ease Inc., Minneapolis, MN, USA). Validation of optimization data is done by comparing the value of the response of the laboratory results with the results of suggestion (prediction) of the Design Expert software version. 7.1.5.Quality of polynomial equation election models indicated by coefficient of determination, R^2 and significance of the regression coefficients determined by F-test and p-value.

Table 2. Yield Response Data and Gel Strength of draft BBD

No	**Parameter Level**			**Extraction Parameter**			**Responses**	
	X_1	**X_2**	**X_3**	**Concentration of HCl (%)**	**Pre-treatment time(hour)**	**Extraction Temperature (^{0}C)**	**Yield (%)**	**Gel Strength (gram bloom)**
1	-1	-1	0	3	12	90	9,06	85,78
2	1	-1	0	7	12	90	8,92	68,58
3	-1	1	0	3	36	90	9,58	308,86
4	1	1	0	7	36	90	8,18	82,92
5	-1	0	-1	3	24	85	7,48	125,82
6	1	0	-1	7	24	85	11,42	243,08

7	-1	0	1	3	24	95	14,82	117,24
8	1	0	1	7	24	95	10,28	62,86
9	0	-1	-1	5	12	85	6,04	62,9
10	0	1	-1	5	36	85	9,8	343,18
11	0	-1	1	5	12	95	9,27	8,06
12	0	1	1	5	36	95	10,86	68,62
13	0	0	0	5	24	90	12,86	294,56
14	0	0	0	5	24	90	12,28	280,26
15	0	0	0	5	24	90	13,7	388,94

III. Results And Discussion

A. Fitting the Model

RSM-BBD in this researchwas used to determine the appropriate model for predicting the response of yield and gel strength of gelatin.The analysis model was used to determine the right model in RSM methodology, which is then used to predict the response to the independent variables [12]. Models were evaluated including linear, 2FI (Interaction), quadratic or cubic.Model selection was carried out by p-value models, lack of fit test and a model summary statistics (R^2, Adjusted R^2 and deviations standard in Table 3.

Model selection by Sequential Model Sum of Squares indicated that the model suggested is significant and quadratic model on the response of yield and gel strength (Table 3). P-value less than 0.05 indicated that the model is significant. Model selection by Sequential Model Sum of Squares on a quadratic model has a p value of 0.0114 (1.14%) and smaller than 0.05 (5%) on the response of yield and p value of 0.031 (3.1%) on the strength gel which showed that the chances of a model error of less than 5%, or mean quadratic model has a significant effect on both response. While based on the lack of fit test, the model will be considered appropriate if the deviation test of the model is not statistically significant at $\alpha = 0.05$ level. If the model has a p-value of more than 0.05, then the model is not significant and is considered in accordance to the response. Program Design Expert DX 7.1.5 showed the quadratic model as the model chosen (suggested) because it has a p-value greater than 5% i.e 26.64% in yield response and 48.76% on the gel strength.

Table 3. Analysis of Variance (ANOVA) for Fitted Quadratic Polynomial Model

Source	Sum of Squares	df	Mean square	F-Value	P-Value Prob>F
Yield					
Model	1592,38	1	1592,38		
Linier	17,62	3	5,87	1,04	0,4141
2FI	19,55	3	6,52	1,22	0,3636
Quadratic	37,27	3	12,42	11,37	0,0114
Qubic	4,44	3	1,48	2,91	0,2664
Residual	1,02	2	0,51		
Lack of Fit	4,44	3	1,48	2,9065	0,2664
Pure Error	1,02	2	0,51		
Cor Total	79,90	14			
R^2 = 0,93 Adjusted R^2 = 0,81 Std.dev =1,05					
Gel Strenght					
Model	4,55E+05	1	4,55E+05		
Linier	60985,91	3	20328,64	1,6	0,246
2FI	39533,31	3	13177,77	1,05	0,4221
Quadratic	81053,68	3	27017,89	6,97	0,031
Qubic	12417,68	3	4139,23	1,19	0,4876
Residual	6974,47	2	3487,24		
Lack of Fit	12417,68	3	4139,23	1,19	0,4876
Pure Error	6974,47	2	3487,24		
Cor Total	2,01E+05	14			
R^2 = 0,90 Adjusted R^2 = 0,73 Std.dev = 62.28					

Quadratic model has the smallest deviation standard compared to the other models in the coefficient of determination (R^2 = 0.93), Adjusted R^2 = 0.81 to increase the yield and R^2 = 0.90), Adjusted R^2 = 0.73 to increase the maximum gel strength i.e 0.93 and 0.81. These data indicated variable concentrations of HCl, pre-treatment time and extraction temperature significantly affected the diversity of the response of 81% and 73% while the rest of 19% and 27% influenced by other factors not be used as variables studied (by value Adjusted R^2).

Optimization of gelatin extraction from bones of catfish using RSM has a value of R^2 = 0.93, P <0.0001, and the lack of fit test (P> 0.05 = 0.1768) indicated that the quadratic model had a significant effect on the response [5].

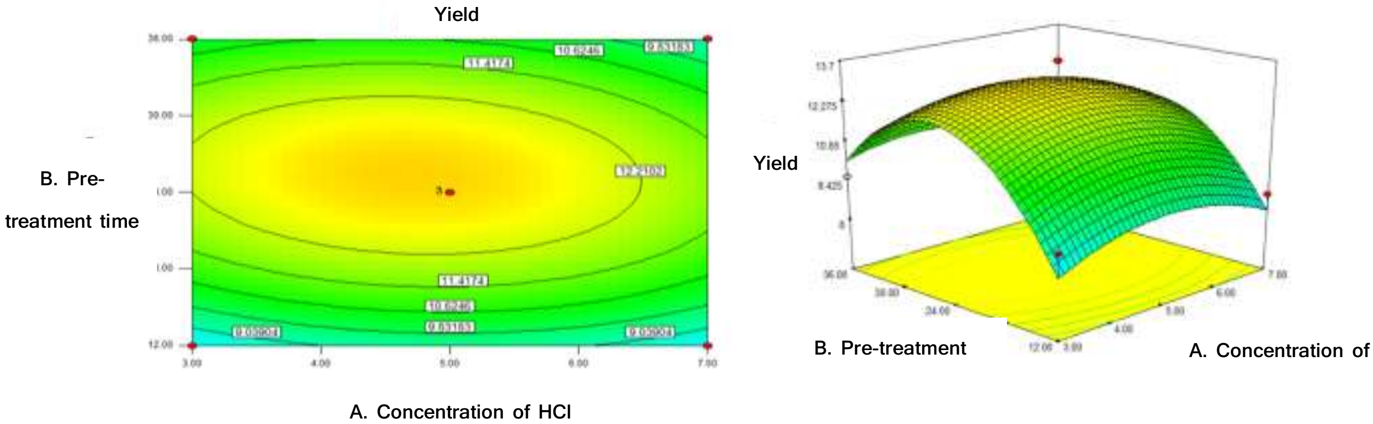
B. Response Surface Analysis

The equation quadratic model was used to predict the response of several levels.Quadratic equation obtained to increase the yield (Y_1) and the gel strength (Y_2) is as follows:

$$Y_1 = 12{,}95 - 0{,}27X_1 + 0{,}64X_2 + 1{,}31X_3 - 0{,}32X_1X_2 - 2{,}12X_1X_3 - 0{,}54X_2X_3 - 1{,}00X_1^2 - 3{,}01X_2^2 - 0{,}94X_3^2$$

$$Y_2 = 321{,}25 - 22{,}53X_1 + 63{,}28X_2 - 55{,}78X_3 - 52{,}19X_1X_2 - 42{,}91X_1X_3 - 72{,}93X_2X_3 - 93{,}08X_1^2 - 91{,}64X_2^2 - 90{,}92X_3^2$$

This study has three variable factors, therefore there are 3 response graphs illustrating the relationship between the concentration of HCl, pre-treatment time and extraction temperature.The influence of the concentration of HCl, pre-treatment time and extraction temperature to increase the yield and gel strength of gelatin contained in Figure 1 and Figure 2 below.



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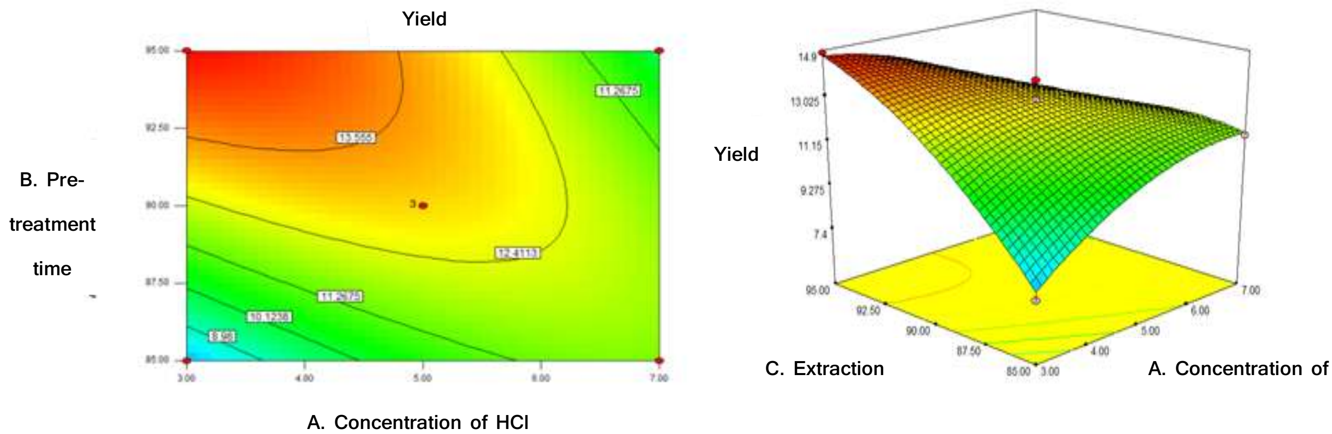
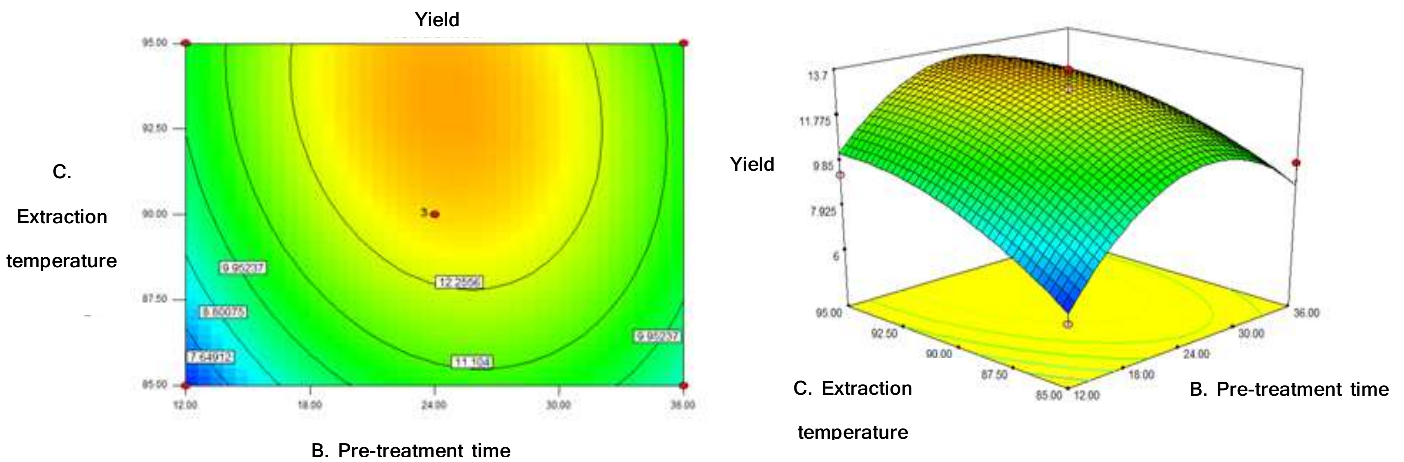
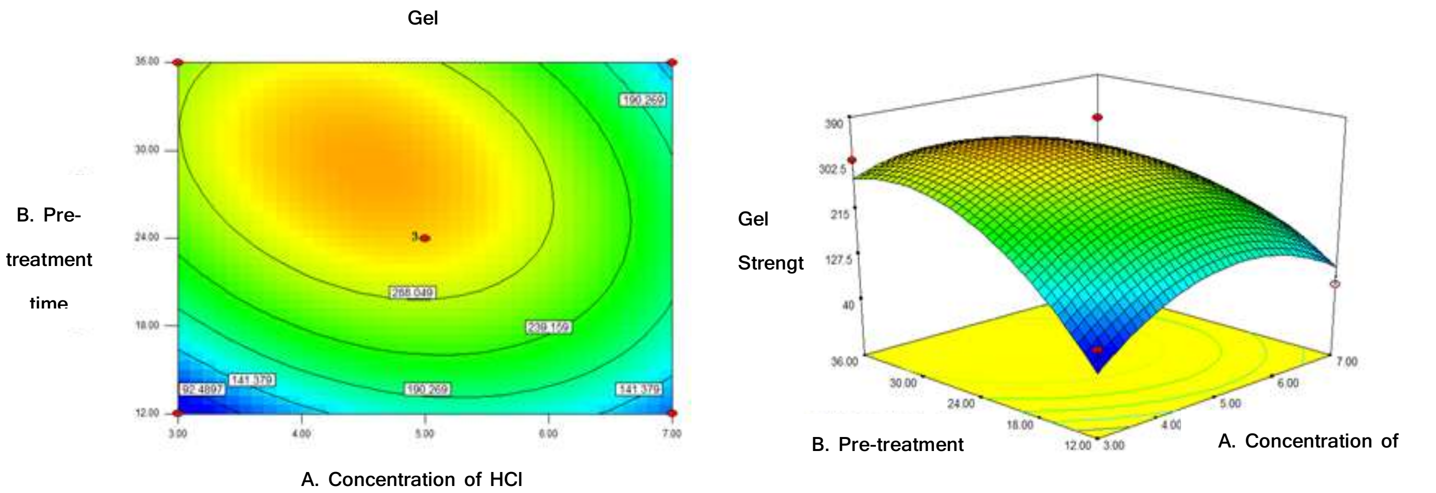


(2)



(3)

Figure 1. Plot Contour and Surface Response Curves (3D) of yield,where (1) the concentration of HCl and pre-treatment time (2) the concentration of HCl and extraction temperatureand (3) pre-treatment time and extraction temperature



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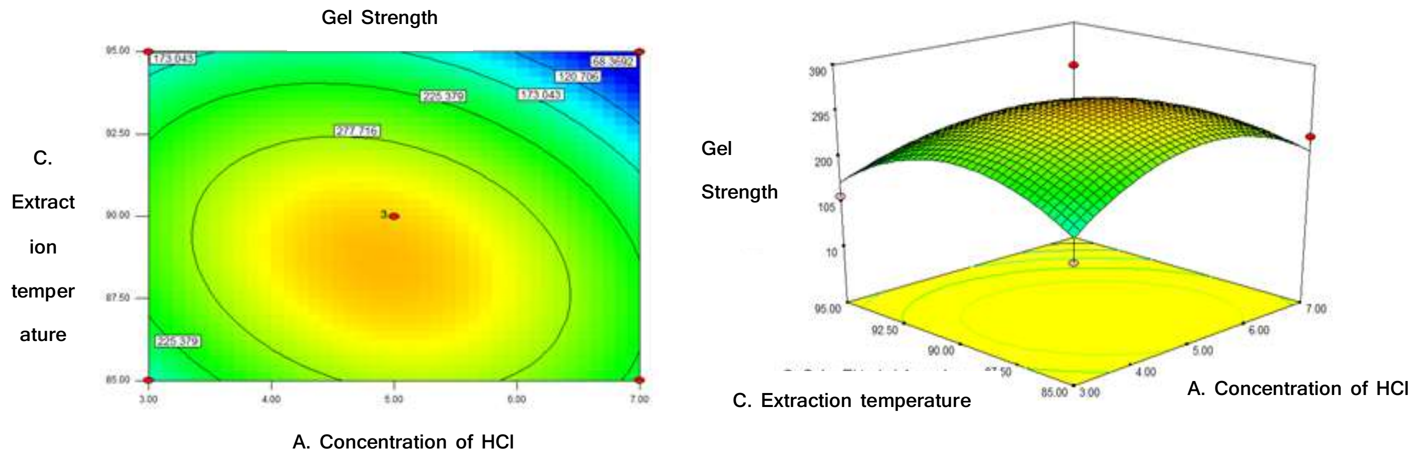

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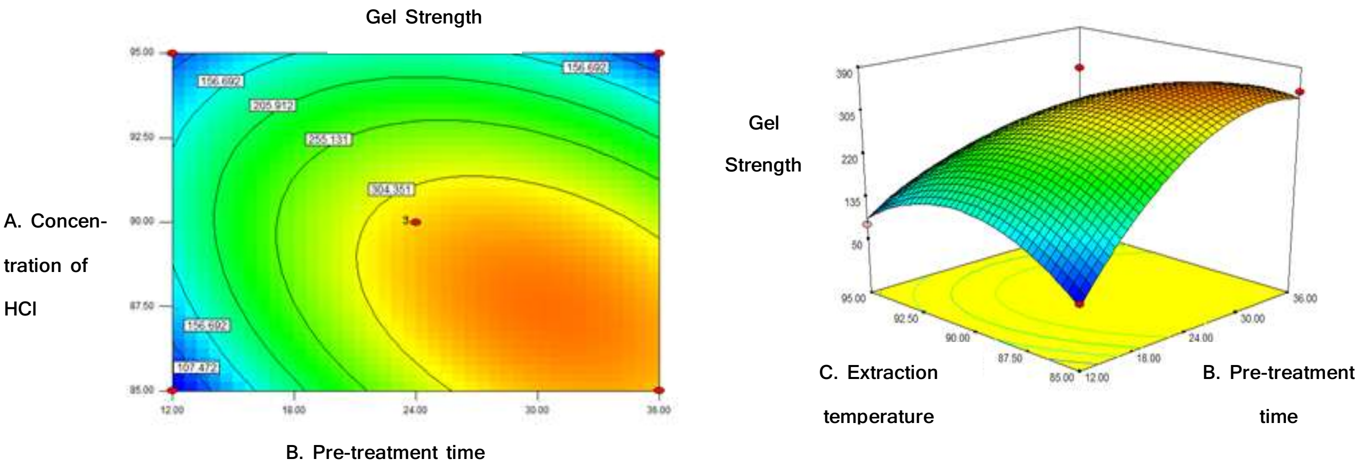

(3)

Figure 2. Plot Contour and Surface Response Curves (3D) of Gel Strength, where (1) the concentration of HCl and Pre-treatment time (2) the concentration of HCl and extraction temperatureand (3) Pre-treatment time and extraction temperature

1. Response Surface Analysis on Yield

Figure 1 shows that the yield reaches a maximum at the level of the range upper and lower limits of each variable. This suggests that the selection of variables on the level of optimization of the extraction conditions are appropriate. The enhancement of the concentrations of HCl (A), pre-treatment time (B) and extraction temperature (C) at the beginning of the extraction up to a certain point will increase the amount of yield, but would decrease if the addition is already too much to the point of saturation.

Yield value is affected by the concentration and extraction time in a solvent of HCl. The higher the concentration and extraction time were more acidic the HCl solvent was, so that H + ions that hydrolyze collagen triple helix of chains become more and more single chain. But the higher the concentration was and the longer the time of extraction was in HCl solvent, the lower gelatin yield would be. This is assumed to occur because of the advance hydrolysis as indicated by the condition ossein that obtained was more softer and easily crushed. At pH neutralization process, along with the washing water soluble ossein which cause the yield decreases. The lysis of the hydrogen bonds and hydrophobic bonds which are the bond stabilizer on the triple helix stabilizer into components α, β, γ will be many more because of the acid concentration and soaking time which were higher so that the conversion of collagen into gelatin become easier and multiplied [13]. Soaking process which was too long will cause collagen solubility so that the yield decreases. The higher amount of HCl concentration gave the more effective hydrolysis effect, namely ossein that formed more optimal and had potential to undergo further gelatin forming and it would be dissolved, then resulting lower gelatin yields on the immersion with the highest concentration of HCl [14].

In general, the higher the temperature of extraction used, then the more gelatin yield obtained. This is because the higher the temperature used, the gelatin extraction process will be more perfect and the resulting amount of gelatin obtained more and more. The higher the temperature of the extraction was, the more and more collagen breaks down into the filtrate [15].High temperatures will help break the hydrogen bonds in the gel hydrolyzed. The number of hydrogen bonds which was broken will facilitate dissolution of collagen in hot water so as to maximize the yield of gelatin [16]. Higher temperatures will cause further hydrolysis so that some gelatin will be degraded and the yield will be reduced.The yield of gelatin decreased presumably happened because the collagen denaturation at high temperatures during the extraction process [17].The higher the temperature and the concentration of excess acid were will lead to further hydrolysis so that some gelatin will also be relegated and it causes a decline in the yield of gelatin [13].

2. Response Surface Analysis on Gel Strength

Figure 2 shows that the gel strength reached a maximum level in the range of upper and lower limits of each variable. This suggests that the selection of variables on the level of optimization of the extraction conditions are appropriate. The escalation concentration of HCl (A), pre-treatment time (B) and extraction temperature (C) at the beginning of the extraction of gelatin to a certain point will increase the strength of milkfish gelatin gel, but would decrease if the addition is already too much to the point of saturation.

Gel formation is influenced by pH, acid concentrations and temperatures. Acid concentration and temperature (heat) will affect the strength of gelatin gel for the structure to be damaged, so that the gel will not be formed [18].The content of α chain and the molecular weight distribution will affect the formation and gel strength.Gel strength decreases along with increasing molecular weight gelatin. Gelatin with larger molecules having a chain connected by covalent bonds. Connective tissue between the molecules is weak because the covalent bond among the chain reduce the number of hydrogen bonds (non-covalent bonding). Gel strength relates to the length of the chain of amino acids which will produce greater gel strength. Optimal hydrolysis will produce long chains of amino acids at the time of conversion of collagen into gelatin to produce high gel strength [19].

C. Determination of Response Optimum Point and Verification

The optimum point obtained from calculation system software Design Expert (Table 4) is concentration variable of 4.65% HCl, pre-treatment time of 26.89 hours and extraction temperature 9.92 ^{0}C.This condition is the optimum condition to obtain fish bone gelatin with maximum yield and gel strength. Verification was done by comparing the value of the actual response of the experimental results with the response value of the calculation soft ware Design Expert. The calculation of the actual experiment obtained the mean of yield response values of 12.97% and 337.46 gram bloom of gel strength while Design Expert from the calculation of yield response values obtained for 12.93% and gel strength of 335.57 grambloom. Verification value and the yield response included in gel strength prediction intervals in Table 5:14 which is in the interval (11.14 to 14.44)% for yield and (245.51 to 425.63) gram bloom for the gel strength.

Table 4.Optimum Solution Point and Verification of Selected Criteria

	IndependentVariable			**Responses**		**Desi-ra-bility**	**Notes**
	Concen-tration of HCl (%)	**Pre-treat-ment time (hour)**	**Extraction Temperature (^{0}C)**	**Yield Respons(%)**	**Gel Strength (gram bloom)**		
prediction*	4,65	26,89	89,92	12,93	335,57	0,81	selected
Interval prediction *	4,65	26,89	89,92	11,14-14,44	245,51-425,63		
Verification**	4,65	26,89	89,92	12,97	337,46		
Differences response value of prediction and verification				0,31%	0,56%		

Information: *Result from Design Expert DX 7.1.5 program

** Actual research data

Differences between the mean value of the yield response and gel strength verification results with calculations of Design Expert respectively 0.31% and 0.56%.The percentage difference in the value of each response is not too large and the value of the verification is nearing the Design Expert calculations so that the difference was considered where the difference in value is not too significant and independent variables solutions provided by Design Expert unacceptable The difference predictive value with the value of the research not more than 5% indicates that the model is quite appropriate [8].

Conclusion

Based on the results of the optimization using RSM Box Behnken Design, it is obtained the conditions for the concentration of HCl as 4.65%, pre-treatment time 26.89 hours and extraction temperature 89.92^{0}C with the results of yield of 12.93% and 335.57 gram bloom of gel strength based on the prediction.Based on the results of verification in laboratory,the amount yield is 12.97% and the gel strength is 337.46 gram bloom.

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