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Extraction and Properties of Gelatin from Spotted Oceanic Triggerfish (*Canthidermis maculata*) Skin and Bone

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***Abstract*— Effects of pretreatments with different alkaline and acid concentrations, and extraction temperatures on fish gelatin extraction and properties was studied. The resultant fish gelatins were evaluated in terms of extraction yield, viscosity, gel strength, and pH. The combination of higher acid pretreatment concentrations and extraction temperatures removed the noncollagenous protein and provided proper pH extraction condition which resulted higher gelatin extraction yield. However, the gel strength decreased with the increase of acid concentration. The gel strength and pH of gelatin increase proportionally to the increase in alkaline concentration and extraction temperature. No significant effects of the three treatments on the gelatin viscocity. The recommended gelatin extraction process conditions was using alkaline concentration (NaOH) of 0.55 N, acid (HCl) of 0.95 N, and a temperature of 60 °C. The optimal solution formula had optimization targets of 79.5% and was predicted to produce a gel with 11.21% yield and pH 3.4**

***Keywords*— Fish gelatin; Alkaline and acid pretreatment; Extraction yield; Gel strength**

I. INTRODUCTION

Gelatin is a polypeptide extracted from collagen tissue found in bones, skin and connective tissue [1]. Utilization of gelatin is quite extensive, both in the food industry as well as non-food industry. In food industry, gelatin is used as a binder agent, stabilizer, gelling agent, adhesives, viscosity agent and emulsifier.

The main sources of gelatin are pig skin (46%), cowhide (29.4%), beef bones (23.1%) and other sources (1.5%) [2]. Unfortunately, muslims cannot consume gelatin from pig skin because it is not lawful, while gelatin derived from cattle is avoided by most people because the fear of bovine spongiform encephalopathy (BSE) disease or mad cow. Since the main source of gelatin is from pig and cow, therefore, it is necessary to find other source of gelatin that is halal for Muslims and safe.

Fish contains collagen which can be extracted to produce gelatin. Collagen in fish is considered as a potential raw material for making gelatin. According to [3], hard bone fish (teleostei) contain collagen ranges from 15% - 17%, while the bone cartilage fish between 22% - 34%. One type of fish that have the potential to be used as raw material for making gelatin is spotted oceanic triggerfish. The fish has very hard skin and bone, therefore only the meat is used, while the rest is discarded without utilization. The fish waste from spotted oceanic triggerfish is up to 51%. Utilization of spotted oceanic triggerfish skin and bone are expected to reduce waste and increase the value of the waste.

This study used Response Surface Methodology (RSM) approach to obtain the optimum conditions of gelatin extraction. RSM is a method that combines mathematical techniques with statistical techniques to create and analyze a response Y (e.g. yield, gel strength, etc.) that are influenced by several variables (independent variable) or factor X [4]. The independent variables used in this study were the concentration of alkaline (NaOH) and acid (HCl) in the pretreatment process, and the temperature of the extraction process. The purpose of this study were to determine the effects of alkaline and acid pretreatments on gelatin extraction, and to obtain optimum gelatin extraction conditions from spotted oceanic triggerfish skin and bone.

II. MATERIALS AND METHODS

Black spotted oceanic triggerfish waste in the form of skins and bones were obtained from the fish auction place in Lampulo Banda Aceh, Indonesia. The fish skins and bones were washed and cleaned from the remnants of meat with knife then packed in plastic bag and stored at -24°C until extraction process. The chemicals used are all based on pro-analysis (PA).

A. Gelatin Extraction in Screening Stage

The raw materials, which were still in frozen state, were cut into small sizes (2-3 cm) and thawed at 4°C overnight. The raw material (20 g) was soaked in a solution of NaOH (1: 5 w / v) with various concentrations (factor a, N) and various soaking times (factor b, h). Then, the samples were washed with water (1: 5 w / v) three times, filtered and then squeezed. Next, the samples were immersed in HCl (1: 5 w / v) with various concentration (factor c, N) and various soaking time (factor d, h), and then the samples were washed with water (1: 5 w / v) three times, filtered and then squeezed. The sample were then put into an Erlenmeyer, added with distilled water (1: 4 w / v) and covered with aluminum foil. Subsequently, the samples were extracted in a water bath with various extraction temperatures (factor e, ° C) and various extraction times (factor f, h). After that, the filtrate was obtained by filtering using cheesecloth and dried in a glass container at a temperature of 60°C for 72 hours.

B. Gelatin Extraction in Optimization Stage

The raw materials (200 g) were soaked in a solution of NaOH (1: 5 w / v) with various concentrations (factor X 1, N) for 1 hour. Then, the samples were washed with water (1: 5 w / v) three times, filtered and then squeezed. Subsequently, the samples were immersed in HCl (1: 5 w / v) with various concentrations (factor X 2, N) for 3 hours, then the samples were washed with water (1: 5 w / v) three times, filtered and then squeezed. After that, the samples were put into glass beakers, added with distilled water (1: 4 w / v) and covered with aluminum foil. Subsequently, the samples were extracted using a water bath with various extraction temperatures (factor X 3, ° C) for 5 hours. Next, the filtrate was obtained by filtering using cheesecloth and dried in a glass container at a temperature of 60°C for 72 hours.

C. Gelatin Yield

The yield is calculated from the ratio of dry weight of gelatin produced over dry weight of the raw materials extracted.

$$Yield = \frac{\text{Dry Weight of Gelatin}}{\text{Dry Weight of Skins or Bones}} \times 100\% \quad (1)$$

D. Gel Strength

The gel strength of gelatin gel was determined according to Gelatin Manufacturers Institute of America standard test method [5] with modification. Gelatin solution (6.67 %, w/v) was prepared by dissolving 7.50 g of gelatin in 105 ml of distilled water in a 250 ml beaker glass (61mm in dia.flat bottom) The beaker was covered and allowed 1-3 hours at room temperature. Then the solution was heated in a water bath for 8-10 minutes at 65 °C with stirring and let at room temperature for 15-20 minutes. After that the beaker was covered tightly and let in water bath for 16-18 hours at 10oC. Gel strength was measured by LFRA Texture Analyzer, using a 12.5 mm plunger, 4 mm penetration into the gelatin gel, speed of 1 mm / sec and trigger 5.

E. Viscosity

The viscosity was measured according to the British Standard 757 1975 whereas gelatin solution (6.67 %, w/v) was prepared by dissolving 7.50 g of gelatin in 105 ml of distilled water in a 250 ml beaker glass which was covered and let for 1-3 hours at room temperature. Then, it was heated in a water bath for 8-10 minutes at 65°C while stirring. After that the beaker was placed on a viscometer instrument and measured when the gelatin solution’s temperature was 6°C with a shear rate of 60 rpm using the spindle 1. The value of viscosity is expressed in units of centipoise (cP).

F. pH

1.6 g of gelatin was put in a glass beaker and added 105 ml of distilled water while stirring. The beaker was covered and allowed 1-3 hours at room temperature. After that, the gelatin solution was heated in water bath for 10-15 minutes at a temperature of 65oC, then into the water bath with temperature of 35°C and pH of the solution was measured while stirring occasionally.

G. Experimental Design on Screening Stage

A fractional factorial design was used for screening (Table 1). There were 6 independent variables selected and one dependent variable was measured as response in screening, i.e. gelatin yield.

TABLE I
INDEPENDENT VARIABLES AND THEIR LEVELS IN THE 6- FACTOR, 2-LEVEL FRACTIONAL FACTORIAL SCREENING DESIGN.

Independent variables	Symbol	Level	
		-1	+1
Alkaline Concentration (N)	A	0.1	1
Alkaline Soaking Time (hours)	b	1	2
Acid Concentration (N)	c	0.1	6
Acid Immersion Time (hours)	d	1	3
Extraction temperature (° C)	E	3	5
Extraction Time (hours)	F	40	60

H. Experimental Design on Optimization Stage

From the results obtained on the screening stage, 3 independent variables were then used in the optimization of extraction of gelatin through Response Surface Methodology (RSM) approach.

TABLE II
THE INDEPENDENT VARIABLES AND THE LEVELS OF INDEPENDENT VARIABLES IN 3 FACTORS AND 5 LEVELS IN CENTRAL COMPOSITE ROTATABLE DESIGN

Independent Variables	Symbol	Code Level				
		-α	-1	0	+1	+α
The concentration of alkaline pretreatment =A (N)	X_1	0.25	0.55	1	1.45	1.75
The concentration of acid pretreatment =B (N)	X_2	0.91	0.95	1	1.05	1.08
Extraction temperature=C (°C)	X_3	33.2	40	50	60	66.9

The independent variables, namely: the concentration of alkaline (X1), the concentration of acid (X2), and the extraction temperature (X3), were suspected as critical variables that can provide significant effects on the extraction of fish gelatin from black spotted oceanic triggerfish waste. Based on these results, the rotatable central composite designs (Tables 2 and 3) were formulated, and four responses evaluated, namely: yield (Y1), gel strength (Y2), viscosity (Y3), and pH (Y4).

I. Analysis of Data

Expert design software version 9.0.5 was used in the optimization of gelatin extraction from waste of black spotted oceanic triggerfish. All data analysis and response surface plot were also done using this software.

III. Results and Discussion

A. Raw Material

The protein content of raw materials of black spotted oceanic triggerfish waste in the form of skin and bone were analyzed by Kjeldahl method. The analysis showed that protein content in the skin and the bone were 29.69% and 28.90% respectively, while the protein content of both bone and skin mixture was 78.38%. Thus, since the bone and skin mixture produces the highest protein content, this raw material was used for the extraction to obtain optimum amount of gelatin yield.

TABLE III

Central composite design for optimizing the extraction of fish gelatin from black spotted oceanic triggerfish in coded unit along with experiment (Exp) and predicted (Pred) results

Run	X_1 (N)	X_2 (N)	X_3 (°C)	Y_1(%)		Y_2(g)		Y_3(cP)		Y_4	
				Exp	Pred	Exp	Pred	Exp	Pred	Exp	Pred
1	+1	-1	+1	10.10	9.51	53.30	63194	1.00	1.235	3.39	3.329
2	0	0	+α	13.83	15.09	28.80	44.669	1.30	1.235	3.31	3.426
3	-1	+1	-1	6.10	6.14	13.00	14.376	1.60	1.235	3.04	2.975
4	0	0	0	6.71	6.14	91.70	38.785	1.70	1.235	2.83	2.932
5	0	0	0	6.98	6.143	57.50	38.785	1.20	1.235	2.92	2.932
6	0	0	0	5.33	6.43	24.70	38.785	1.20	1.235	2.73	2.932
7	+α	0	0	4.24	4.851	66.00	57.177	1.10	1.235	2.98	3.148
8	-1	-1	+1	11.82	11.212	47.70	41.322	1.20	1.235	3.44	3.400
9	0	0	-α	3.91	3.424	19.30	32.901	1.00	1.235	3.09	3.153
10	0	0	0	3.80	6.143	82.30	38.785	1.30	1.235	3.18	2.932
11	-α	0	0	8.78	8.943	11.80	20.393	1.40	1.235	3.02	3.031
12	+1	+1	+1	11.45	10.567	37.00	43.245	1.00	1.235	3.43	3.276
13	0	0	0	6.20	6.143	7.20	38.785	1.20	1.235	2.91	2.932
14	0	-α	0	5.89	6.173	28.30	55.560	1.50	1.235	3.23	3.263
15	0	0	0	7.97	6.143	56.00	38.785	1.10	1.235	3.05	2.932
16	+1	+1	-1	2.91	2.971	19.80	36.248	1.30	1.235	3.27	3.184
17	+1	-1	-1	3.57	3.508	65.30	56.197	1.20	1.235	3.21	3.127
18	-1	-1	-1	4.60	4.936	32.30	34.325	1.10	1.235	3.14	3.167
19	0	+α	0	7.58	8.070	9.50	22.010	1.20	1.235	2.91	3.056
20	-1	+1	+1	14.49	14.005	24.20	21.373	1.10	1.235	3.14	3.097

(X1=the concentration of pretreatment alkaline (N), X2=the concentration of acid pretreatment (N), X3=the extraction temperature, Y1=yield, Y2=gel strength, Y3=viscosity, and Y4=pH.

B. Screening

Based on the results of previous study [6] and the experiments in the screening stage, it was concluded that the alkaline pretreatment concentration (A=X_1, N), acid pretreatment concentration (B=X_2, N), and the extraction temperature (C=X_3, °C) were significant factors affecting gelatin yield (Table 2). Previous research associated with the extraction of gelatin, [7], [8], [9], as well as [10] has also concluded that the concentration of alkaline, acid, and the extraction temperature affect the yield and quality of fish gelatin.

C. Response Surface Model Building of Gelatin Extraction

On the optimization of the gelatin extraction, the response variables optimized were yield, gel strength, viscosity, and pH. Table 3 shows the experimental results of 3-factor, 5 level central composite design with the independent variables (X) and the experimental responses along with the predictions (Y) . The response surface models for every response investigated showed in Table 4.

TABLE IV

Response Surface Model for Yield, Gel Strength, Viscosity, and pH Responses

Respon	Model	Significant ($p<0.05$)	Lack of fit ($p<0.05$)	R^2
Yield	Quadratic	< 0.0001	0.8197	0.8742
Gel strength	Linear	0.1597	0.9624	0.0655
Viscocity	Mean	-	0.6957	< 0.0001
pH	Quadratic	0.1024	0.5305	0.3857

D. Yield

The gelatin yield of bone and skin of black spotted oceanic triggerfish ranged from 2.91% to 14.49%, with an average yield of 7.31%. The results of analysis of variance (ANOVA) showed that the chosen model for the yield response was quadratic (Table 4), because this model had higher value of R2 than other models, i.e. 0.87. This model was significant (P $\leq$ 0.05) for the yield. The results from ANOVA also showed that the alkaline pretreatment concentration (A=X1, N) and the extraction temperature (C=X3, °C) were significant factors affecting the yield, while the acid pretreatment concentration (B=X2, N), the interaction between factors, and Lack of Fit effects were not significant (P≥0.05). According to [11], an insignificant value of Lack of Fit is a requirement for a good model, since it indicates conformity between the yield response data and the model.

The RSM equation of the gelatin extraction optimization towards the yield response is as follows:

$$\text{Yield} = +6.14 - 1.22\,A + 0.56\,B + 3.47\,C - 0.43\,AB - 0.067\,AC + 0.40\,BC + 0.27\,A^2 + 0.35\,B^2 + 1.10\,C^2 \quad (2)$$

where A, B, C as in Table 2, i.e:

A: The concentration of alkaline pretreatment
B: The concentration of acid pretreatment
C: The extraction temperature

This equation showed that the yield response will increase proportionally with the concentration of acid pretreatment and the extraction temperature which are indicated by constant positive values. This presumably because acid produced a pH that was suitable for the extraction of gelatin and also acid caused some cross-linkages easily to break with little damage to the polypeptide chain [9]. Therefore, a higher yield could be generated. Extraction temperature also affected the yield, based on the statement of [12], during extraction; triple helix molecules will lose stability and break down into three α chain, and upon denaturation of collagen, the triple helix chain completely transformed into a single chain of gelatin. Therefore, the higher the temperature the easier the extraction of triple helix chain, consequently the higher the yield of the resulting gelatin.

Fig. 1 shows normal plot of residuals graph of yield response, which identifies the relationship between the actual value and predicted value. It shows that data for yield response spread normally approaches normality line. This means that the actual results will be close to the predicted results from the DX 9.0.5 program.

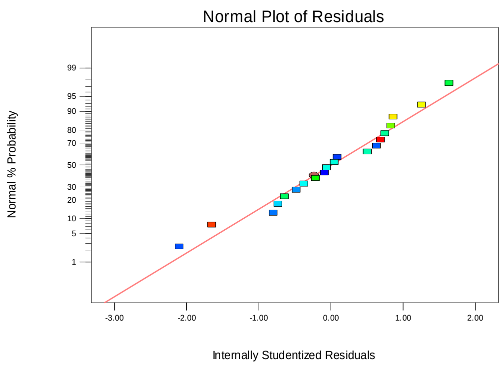

Fig. 1. Normal plot of residuals of yield response.

Contour plot graph in Fig. 2 shows how the combination between components (X_1, X_2, X_3) interplay yield response value. Different colors on the graph contour plot indicate different yield values. Blue indicates the lowest yield response value and red indicates the highest yield response. The lines which composed of dots on the contour plot graph represent the combination of three components with different values which produced similar yield response.

Surface shape of interaction between these components can be seen clearly on three-dimensional graph illustrated in Fig. 3.

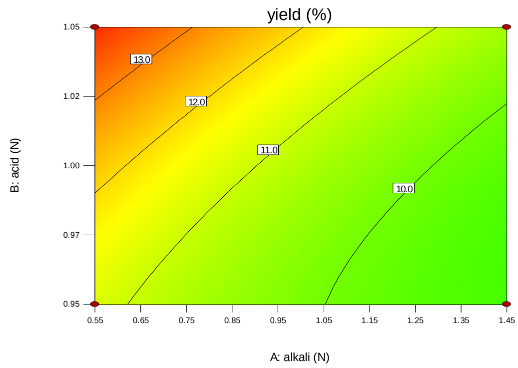

Fig. 2. The response surface plots of gelatin yield of black spotted oceanic triggerfish waste

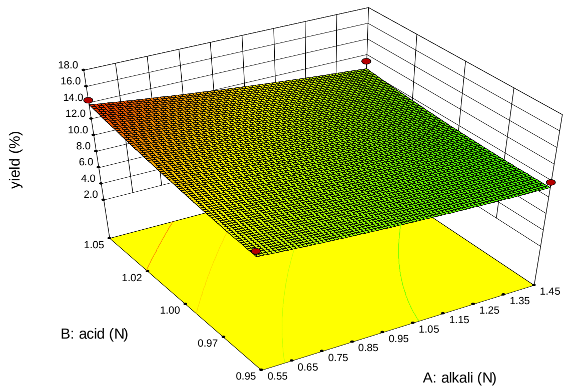

Fig. 3. Surface profile plot of yield response.

E. Gel Strength

The gel strength is measured the strength of the gel on the surface of the sample with a distance of 4 mm and at a concentration of 6.67%. The gel strength of skin and bone gelatin of black spotted oceanic triggerfish ranged between 7.20 grams to 91.70 grams with the average value of 38.78 grams. Results of analysis of variance (ANOVA) showed that the model chosen for the gel strength of the response was linear (Table 4), because this model had the highest value of R2 with a value of 0.0655. This model was insignificant (P≥0.05) because the value was 0.1597. The treatments of concentrations of alkaline pretreatment, concentration of acid pretreatment and extraction temperature also showed insignificant effect on the gel strength response.

RSM equation for extraction optimization of gelatin towards gel strength response is as follows:

$$\text{Gel strength } Y = +38.79 + 10.94\,A - 9.97\,B + 3.50\,C \quad (3)$$

where: A, B, C as in Table 2.

The equation shows that the gel strength response will increase proportionally to the increase in the concentration of alkaline and the extraction temperature which indicated by the constant positive values. However, acid concentration showed a different response. An increase in acid concentration will result in the decrease of gelatin gel strength response.

Normal plot of residuals graph that identifies the relationship between the actual and the predicted values in the data to indicate the response that spreads the gel strength was not close to the normal line (Fig. 4). This means that the actual results were not close to the results predicted by the DX 9.0.5 program.

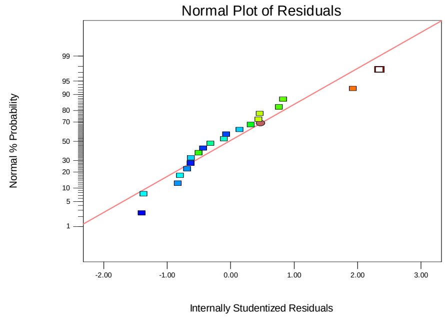

Fig. 4 Normal plot of residuals of gel strength response

Contour plot graph in Fig. 5 shows how the combination between components (X1, X2, X3) interplay gel strength response value. As for Fig. 2, the different colors on the contour plot graph indicate different gel strength values. The blue, red, and dots on the graph indicated the same information as in Fig. 2. The three-dimensional graph of the surface shape of interaction between these components is illustrated in Fig. 6.

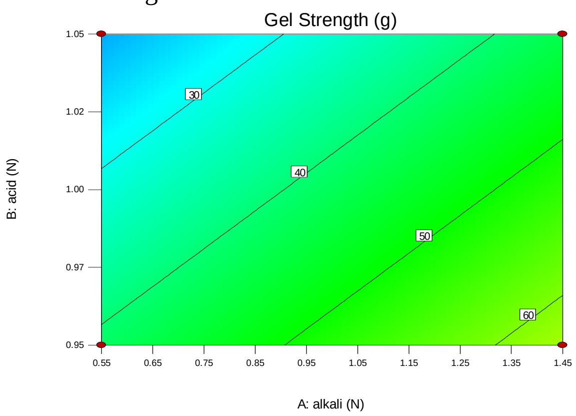

Fig. 5 The response surface plots of gel strength

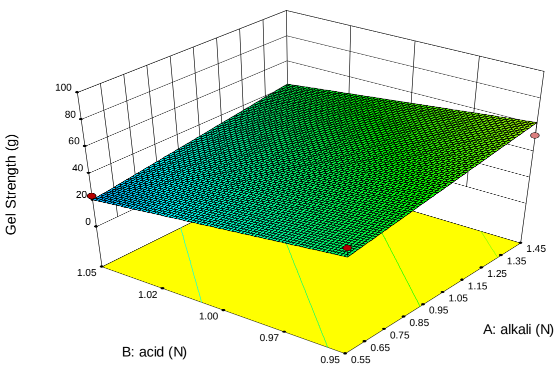

Fig. 6 Surface profile plot of gel strength response.

F. Viscosity

Gelatin viscosity testing was conducted to determine the level of viscosity of the gelatin as a solution at a certain concentration and temperature. According to [13], the viscosity of the gelatin is usually measured at a temperature of 60 ^{o} C. The gelatin viscosity of skin and bone of black spotted oceanic triggerfish ranged from 1.00 cP to 1.70 cP with an average value of 1.2 cP. Results of analysis of variance (ANOVA) showed that the model chosen for the response of viscosity was the average (mean) (Table 4), because other models had negative R^2 values. The model indicated no significant effect (P≥0.05) and all of the independent variables (X_1, X_2, X_3), i.e. alkaline and acid pretreatment concentrations, and extraction temperatures also showed no significant effects on the viscosity response.

Normal plot of residuals in Fig. 7 identifies the relationship between the actual and predicted values which shows that the data were spread away from the normal line. This means that the actual results were not approached the results predicted by the program DX 9.0.5.

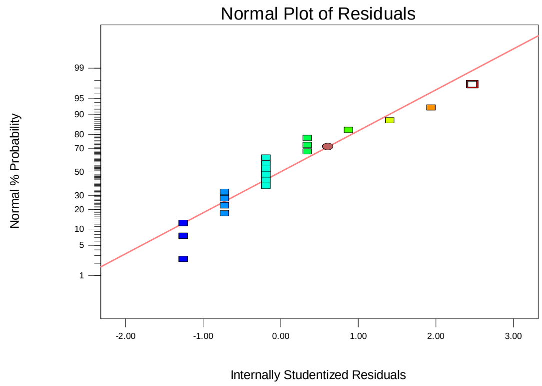

Fig. 7 Normal plot of residuals of viscosity response

G. The Degree of Acidity (pH)

The pH value of the gelatin is influenced by pretreatment of raw material for extraction [14]. The pH value of gelatin ranged from 2.73 to 3.44 with an average value of 3.10. Results from analysis of variance (ANOVA) showed that the models were chosen for pH response was quadratic (Table 4), because this model had the highest value of R^2 compared to other models with value of 0.3857. This model had no significant effects (P≥0.05). Likewise, all of the independent variables showed no significant effect on the pH response. The equation for the pH response was:

$$pH = +2.93 + 0.035A - 0.061B + 0.081C + 0.063AB - 7.500E{-003}\ AC - 0027\ BC + 0056\ A^2 + 0081\ B^2 + 0.13\ C^2 \qquad (4)$$

where: A, B, C as in Table 2.

The equation shows that pH response will increase proportional to the increase in the concentration of alkaline and the extraction temperature which indicated by the constant positive value. Acid concentrations showed an opposite effect, where an increased in acid concentration will decrease the pH of the gelatin. Normal plot of residuals chart (Fig. 8) shows the data for the pH response spread a little bit out of the normal line. This means that the actual results are not too close to the results predicted by the program DX 9.0.5.

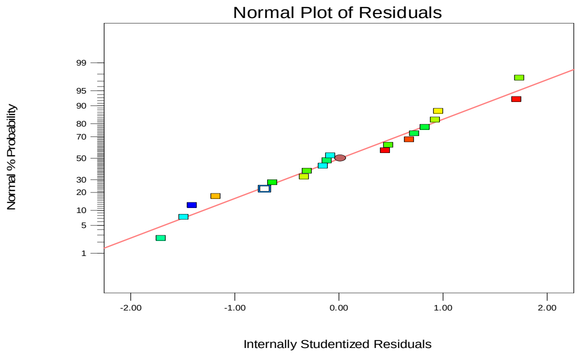

Fig. 8 Normal plot of residuals of pH response

Contour plot in Fig. 9, shows how the combination between components interplay response of the pH value. Different color on the chart contour plot shows a different value. The green color indicates the value is low and a reddish yellow color indicates a higher pH response. The lines which are composed of dots on the graph contour plot show the combination of the three independent variables with different values that produces the same pH response. The surface shape of the interaction between these components can be seen more clearly in the three-dimensional graph shown in Fig.10.

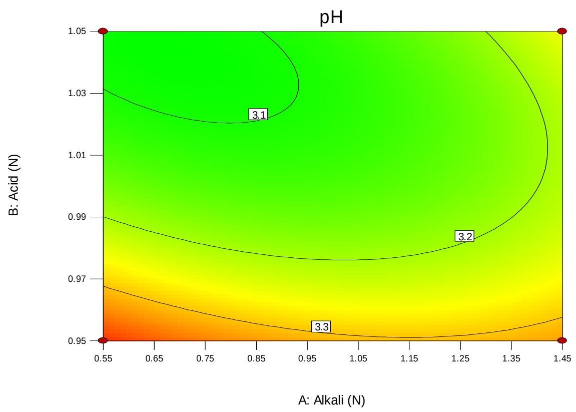

Fig. 9 The response surface plots of pH of gelatin

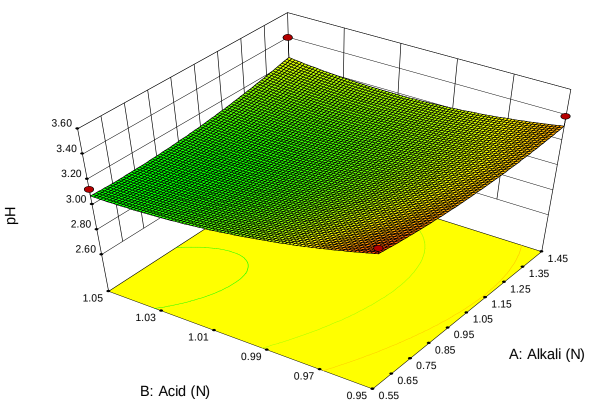

Fig. 10 Surface profile plot of pH response.

H. Gelatin Extraction Optimization

Optimization was done after the mathematical model obtained for each response and was made to obtain desired responses. The purpose of optimization was to minimize the effort required and maximize the desired results. Through this approach, optimization of multi responses could be obtained using desirability functions [15]. Table 5 shows the components which were optimized, targeted, limits, as well as the rate of interest at the stage of formulating the optimization.

The concentration of alkaline and acid, and extraction temperature will affect the quality of the resulting gelatin. Yield and pH responses were responses that would be optimized. These responses had the same models that were quadratic, whereas the gel strength and viscosity responses had linear models, and both the means and the models were not significant therefore were not included in the optimization. Yield response was optimized with the target maximum interest rate of 5 (+++++). Yield was a response which will determine the efficiency of the gelatin extraction process. The better the gelatin extraction process, the higher the gelatin yield, whereas pH was optimized with maximum target and with interest rate of 3 (+++).

TABLE V
OPTIMIZATION PARAMETERS IN THE RESPONSE OPTIMIZER

Component/ Response	Target	Lower Limit	Upper Limit	Importance
Alkaline Concentration	In range	0.55	1.45	3(+++)
Acid Concentration	In range	0.95	1.05	3(+++)
Extraction Temperature	In range	40	60	3(+++)
Yield	Maximum	2.91	14.49	5(+++++)
pH	In Target	2.73	3.44	3(+++)

There were 72 optimization solution formulas obtained from the optimization process and only 1/8 optimization formulas preferred which had higher desirability values (Table 6).

TABLE VI
FORMULA GENERATED IN THE OPTIMIZATION PHASE

No	Alkaline (N)	Acid (N)	Temperature (°C)	Yield (%)	pH	Desirability
1	0.550	0.950	60.000	11.212	3.400	0.795
2	0.558	0.951	60.000	11.195	3.393	0.791
3	0.550	0.950	59.899	11.158	3.396	0.790
4	0.550	0.952	59.986	11.238	3.385	0.790
5	0.550	0.954	60.000	11.269	3.376	0.787
6	0.550	0.950	59.815	11.115	3.393	0.786
7	0.590	0.950	60.000	11.091	3.387	0.782
8	0.550	0.954	59.847	11.183	3.372	0.781
9	0.599	0.950	60.000	11.064	3.384	0.779

Gelatin extraction process conditions recommended was using alkaline concentration (NaOH) of 0.55 N, acid (HCl) of 0.95 N, and a temperature of 60 °C. The optimal solution formula had optimization targets of 79.5% and was predicted to produce a gel with 11.21% yield and pH 3.4

Fig. 11 and 12, describes the optimization results in two and three dimensional (2D and 3D) contour forms. Two-dimensional contour plot (Fig. 11) shows the responses of the predictive models for gelatin yields and pH values. The lines which were composed of dots shows the combination of the three components with different values that produces a certain desirability same value. Prediction point in the figure shows the combination of alkaline concentration (NaOH) of 0.55 N, acid (HCl) of 0.95 N, and a temperature of 60°C that produced desirability value of 0.795. In addition, the three-dimensional graph (Fig. 12) shows the projection of the contour plots. The higher the area, the higher the desirability value, and vice versa if the area is low, the desirability will show a low value.

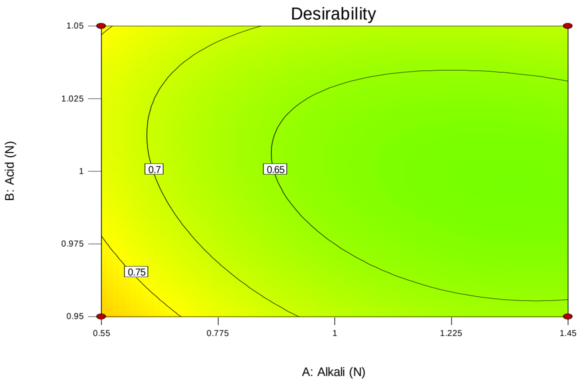

Fig. 11 Contour plot with optimum desirability formula

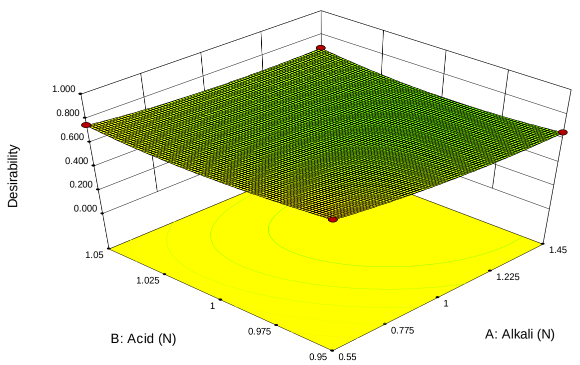

Fig. 12 Three dimensional surface profile plot with optimum desirability formula

IV. Conclusions

An increased in alkaline concentration and extraction temperature caused a decreased on yield, and increased on gel strength and pH, whereas an increase in acid concentration and temperature led to an increase in yield. The viscosity was not affected by the concentration of alkaline, acid and temperature. Optimization using expert design program 9.0.5 via response surface methodology approach generates optimal formula for gelatin extraction with alkaline concentration (NaOH) of 0.55 N, acid (HCl) of 0.95 N and a temperature of 60°C with desirability value of 0.795.

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