Chicken Feathers Based Keratin Extraction Process Data Analysis Using Response Surface-Box-Behnken Design Method and Characterization of Keratin Product

Kidus Tekleab Welu[1], Surafel M. Beyan[1], Subramanian Balakrishnan[1] and Habtamu Admassu[2]*

[1]Department of Chemical Engineering, College of Biological and Chemical Engineering, Addis Ababa Science and Technology University, Addis Ababa, Ethiopia
[2]Department of Food Process Engineering, College of Biological and Chemical Engineering, Addis Ababa Science and Technology University, Addis Ababa, Ethiopia

Received: 24 October 2019, Revised: 17 January 2020, Accepted: 31 January 2020

Abstract

In the process of production of chicken poultry meat for human consumption, the discarded feather is a solid waste management problem worldwide. There are also millions of tons of feather waste every year from the poultry meat industry in Ethiopia. In general, from this waste feather, keratin constitutes to about 91% which could be augmented for further value-added products such as basic nutrient, medical substance and fertilizer. Hence, this present research is carried out to optimize the extraction and characterization of keratin protein from the waste chicken feather. Keratin extraction using Na_2S as a reducing agent was studied by response surface methodology using the Box-Behnken method. This data analysis is applied for the keratin extraction process to study the effect of the most significant factors such as reducing agent concentration, extraction time and mixing ratio. Applying response surface methodology, a maximum yield of keratin reached 75.39% at sodium sulfide concentration of 0.43 M, extraction time of 5.43 h and mixing ratio of 26.65 g/l. Keratin protein product was characterized using Fourier Transform Infrared spectroscopy (FTIR), UV-Visible spectroscopy, scanning electron microscopy (SEM), and X-Ray Diffraction (XRD). The analysis by FTIR confirmed the presence of chemical compositions such as carboxyl acid and amino groups in the protein samples. The surface morphology studied by scanning electron microscopy analysis showed the formation of porosity and aggregate which were expected on the powder of keratin. Structural studies carried out by X-ray diffraction suggest that sodium sulfide stabilized the β-sheet structure of the protein.

Keywords: chicken feather, keratin protein, sodium sulfide, RSM-BBD
DOI 10.14456/cast.2020.6

*Corresponding author: Tel.: +25 191 321 7366
Email: hadtess2009@gmail.com

1. Introduction

A keratinous protein of waste chicken feather has created much attention in recent years. It is the foremost and richest composition of fibrous protein found in chicken feathers, hair, skins, bristles, horns, and hooves [1, 2]. Principally chicken feather contains 91% keratin, which has high mechanical strength [3, 4]. Approximately, billion tons of keratin containing wastes are generated worldwide from the poultry slaughterhouse and wool textile industry every year [5, 6]. Since 5% of the bodyweight of poultry bird is feathers, about three tons of feather waste per day can easily be produced from 50,000 chickens in the slaughterhouse [7]. Chicken feather waste can cause a risk to human health and environmental pollution, therefore, chicken feather discarded in the course of the production of poultry meat for human usage can be a huge problem [8]. There is scant demand for waste chicken feather. Approximately about five billion tons of chicken feather scraps generated annually worldwide by poultry meat producers which are currently disposed by way of landfill or burning or grinding them up to produce farm animals' fodder supplement. Further, feather burnings in special installations are economically expensive [6]. Nowadays, there is a growing interest in manufacturing of value-added materials that are economical and produced from this scrap and renewable resources. Proteins are polymers formed by way of polymerizing different amino acids and the ability to foster intra- and inter-molecular secondary bonds allowing and resulting materials to have a significant difference in their functional groups [7]. Chicken feathers discarded during poultry meat production for human consumption have become a seriously solid and agricultural waste problem world-wide since this waste generates greenhouse gases and also poses danger to the living [8]. There are millions of tons of feather waste generated out of the poultry meat industry in Ethiopia, and it may pose risk to human health and to the environment [3]. The main objectives of this study were to optimize the keratin extraction process using RSM and the characterization of this keratinous protein product.

2. Materials and Methods

2.1 Materials

The feathers of chicken were gotten from Elefora Agro-industries PLC, Debrezeyit, Ethiopia. Sodium sulfide (2M), sodium hydroxide and ammonium sulfate (98%) were acquired from Sigma-Aldrich (St. Louis, MO), USA and the rest of the chemicals used in this research are analytical reagent grade. Products are characterized using the following equipment: UV-visible spectroscopy (CSA C22.2 No 61010-1), Fourier Transform Infrared Spectroscopy (FTIR, PerkinElmer Spectrum IR Version 10.6.1), Scanning Electron Microscope (SEM FEI, INSPCT-F50, and Germany) and X-ray diffractometer (XRD).

2.2 Pre-treatment and keratin extraction

The raw feather was cleaned by soaking in diethyl ether and washed with detergent and dried in an oven at 50^{o}C for 24 h, then it was ground. By this technique, feather sample was cleaned from stains, oil, and grease, etc. The specifics of this method are described by Rouse *et al*. [9] and Sharma *et al*. [10]. Finally, it was stored in a clean closed container at ambient temperature for further study.

2.3 Extraction process optimization using Box Behnken Design (BBD) method

The Box Behnken design is one of the most widely used experimental designs for the Response Surface Methodology (RSM) approach. This is found to be an effective design for sequential experimentation as it provides a reasonable amount of information to test the lack of fit with the required number of experimental values [11]. Prior to following BBD experiments, the levels and chosen parameters for the BBD experiments (mixing ration, extraction time and sodium sulfide concentration) were chosen by results obtained from experimental studies elsewhere through literature survey [12, 13] (Table 1). Sodium sulfide solution at different concentrations, 0.2 M, 0.4 M, and 0.6 M, were prepared using 1l conical flasks. Powdered feathers (20, 25 and 30 g) were added to solution of sodium sulfide. The solutions were continuously mixed for different times (4, 5 and 6 h) at ambient temperature and pH was maintained in the range of 10-13 (alkaline ranges). The solution was filtered and then separated using a centrifuge (10,000 rpm for 5 min) [14]. Then BBD was applied for these experiments. Based on the number of factors and levels required, seventeen experimental runs were conducted to use BBD for the extraction process optimization aided by Design-Expert software 7.0.0 (Stat-Ease, Inc., Minneapolis). To check the adequacy of the variance analysis model (ANOVA) was used. ANOVA was used to obtain desired responses during the course of the experiments. The model used to fit the results of the three-level design is represented by equation 1:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} b_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \varepsilon \quad (1)$$

where X_1, X_2,…, X_n are the input factors that can influence the response Y; n is the number of variables, b_0 is the constant, b_{ii} (i = 1, 2,…n) is the quadratic coefficient, b_{ij} (i = 1, 2,…., n; j = 1, 2,…, n) is the interaction of the coefficient, and ε represents the random error.

Table 1. Levels of variables tested in Box Behnken Design (BBD) for optimization of keratin extraction

Parameters	**Units**	**-1**	**0**	**+1**
Sodium sulfide concentration	M	0.2	0.4	0.6
Extraction time	(h)	4	5	6
Mxing ratio	(g/l)	20	25	30

2.4 Protein precipitation and purification

The feather filtrate solution obtained was transferred into a beaker and then stirred. The solution of ammonium sulphate has been carefully added dropwise [14]. Consequently, the 1:1 ratio of the solution for filtrate and ammonium sulphate was reached. At 4°C, the above solution was subjected to a centrifuge for 5 min at 10,000 rpm and solid particles were stored. With the modification to the method of Shah *et al*. [15], the solid particles were gathered and mixed with de-ionized water till the volume of 100 ml was obtained. The solution was centrifuged at 10,000 rpm for the time duration of 5 min and the solid particles were stored. In 100 ml of 2 M solution of sodium hydroxide, the obtained solid particles were further dissolved. The solution was centrifuged again at 10,000 rpm for 5 min at 4°C and the total liquids were collected and kept for further analysis. Usually, phases of precipitation, washing and dissolution are repeated. The

keratin was precipitated by the isoelectric point that was adjusted with a pH of 3.5 to 4.5 [16, 17]. Finally, it was powdered by a freeze dryer at a temperature of -30ºC for 5 h.

2.5 Characterization of keratin

2.5.1 UV- visible spectroscopy

Freeze-dried keratin was dissolved in distilled water and the solution was analyzed with UV-visible spectrophotometer (CSA C22.2 No 61010-1). The wavelength range was set between 200 nm to 400 nm to determine the desired composition of keratin protein.

2.5.2 Fourier transform infrared spectroscopy (FTIR)

Partially purified freeze-dried keratin powder was used for Fourier Transform Infrared spectroscopy (FTIR) analysis to study functional groups of the products. FTIR run was made between 4000 and 400 cm^{-1} wavenumber range. KBr pellet method was used for performing FTIR analysis.

2.5.3 Scanning electron microscope (SEM)

The morphological structure of the partially purified keratin was studied by using a scanning electron microscope (SEM), model FEI, INSPCT-F50, Germany. The freeze-dried films were fixed on aluminum stubs, coated with a carbon conductive tape and visualized by SEM, operating at 8 kV in a vacuum, and used for identifying the shape and surface morphology of keratin.

2.5.4 X-ray diffraction (XRD)

XRD analysis of keratin was executed using a multi-purpose X-ray diffractometer equipped with a diffraction beam of monochromatic, and a copper marked X-ray tube operated at 0.1 s per step scanning speed to identify the α- helix and β-sheet arrangements in product keratin powder. The data within the scattering angle ranges of 5º to 50º were recorded.

3. Results and Discussion

3.1 Box Behnken Design (BBD) and Response Surface Analysis

Seventeen experiments with a different selection of factor combinations were performed. The experimental and responses of predicted values are given in Table 2. The effects of the factors for all model terms were analyzed by ANOVA. P-values, lack of fit, and R^2-values were used for comparing the models, and accordingly, from a number of possible models, a quadratic model was found to be suitable for the estimation of the given yield as shown by the equation 2 where all variables are given through the coded values.

$$Yield\ (Y) = +74.98 + 1.52 \times A + 0.81 \times B + 0.67 \times C + 0.21 \times A \times B + 2.7 \times B \times C - 5.36 \times A^2 - 1.99 \times B^2 - 2.82 \times C^2 \quad (2)$$

Where, A- Sodium sulfide concentration, B- extraction time and C- mixing ratio.

Table 2. BBD matrix of independent variables used in RSM with corresponding experimental and predicted values of the response

Run no.	Factor 1 A: Concentration of sodium sulfide (M)	Factor 2 B: Extraction time (h)	Factor 3 C: Mixing ratio (g/l)	Yield % Actual (Experimental yield)	Predicted
1	0.2	4	25.00	65.27	65.51
6	0.6	5	20.00	67.45	67.56
8	0.6	5	30.00	68.68	69.09
14	0.4	5	25.00	75.21	74.98
10	0.4	6	20.00	67.48	67.62
3	0.2	6	25.00	66.44	66.71
11	0.4	4	30.00	67.48	67.34
15	0.4	5	25.00	75.45	74.98
17	0.4	5	25.00	73.79	74.98
2	0.6	4	25.00	68.40	68.13
12	0.4	6	30.00	74.52	74.36
13	0.4	5	25.00	75.38	74.98
7	0.2	5	30.00	65.98	65.87
4	0.6	6	25.00	70.41	70.17
9	0.4	4	20.00	71.23	71.39
5	0.2	5	20.00	65.11	64.70
16	0.4	5	25.00	75.09	74.98

3.2 Analysis of variance

The above results were investigated and the calculated determination of coefficient (R^2) for keratin extraction from waste chicken feather was 0.9896 indicating that 98.96% of the response variability could be understood and explained by the statistical model and the model had limitation for extent of 1.04% of the total variation. This implies that the expected values were closer to experimental data and the quadratic polynomial can represent the process for the given experimental domain. The modified R^2 value corrects the R^2 value for the total of factors in the model. The model was found to be highly significant in analyzing the data since the adjusted determination coefficient (Adj R^2 = 0.9761) was found to be very high [18]. "Adeq Precision" determines the signal to noise ratio and if the ratio is higher than four, the model is appropriate. For this study, the ratio of 22.117 showed a tolerable model. Therefore, this model could be useful to navigate the design space. The smaller value of the variation of coefficient, CV= 0.86% indicates the precision with which the experiments were conducted. Similarly, the smaller predicted PRESS statistic shows the better data points fitted the model. Normal probability plots are also a suitable graphical method for judging the normality of the residuals. Residuals normal plot between the normal probability (%) and the internal studentized residuals were obtained to

determine the model approval assumption of analysis of variance. The internally studentized residuals are used to calculate the standard deviations between the experimental and predicted values. Figure 1 showed the relationship between the normal probability (%) and the internal studentized residuals. The straight-line plot indicates that no response variable transformation was required and also apparently there was no problem with normality. The near data straight-line obtained means the residuals were normally distributed and the results were not substantially affected by departures from normality. The predicted values given by a normal distribution as shown in Figure 2 are also plotted against the residual observed, and the points are close to the fitted line that showed a good fit.

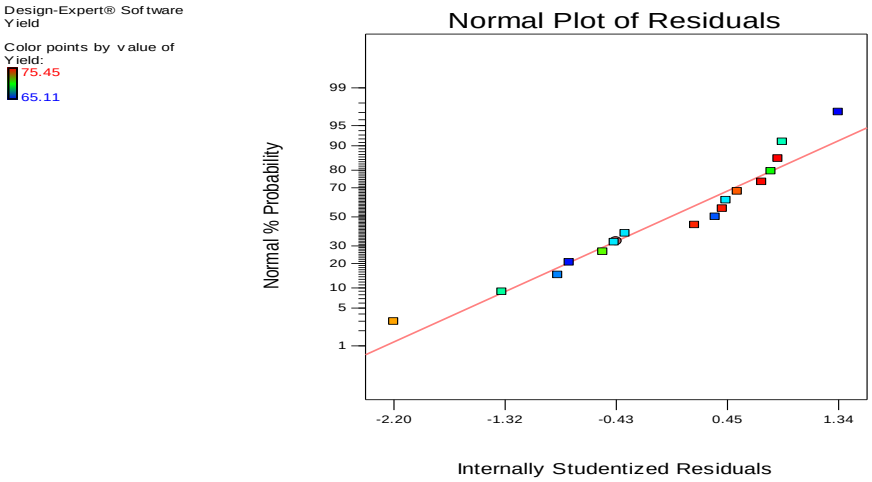

Figure 1. Normal plot of residuals

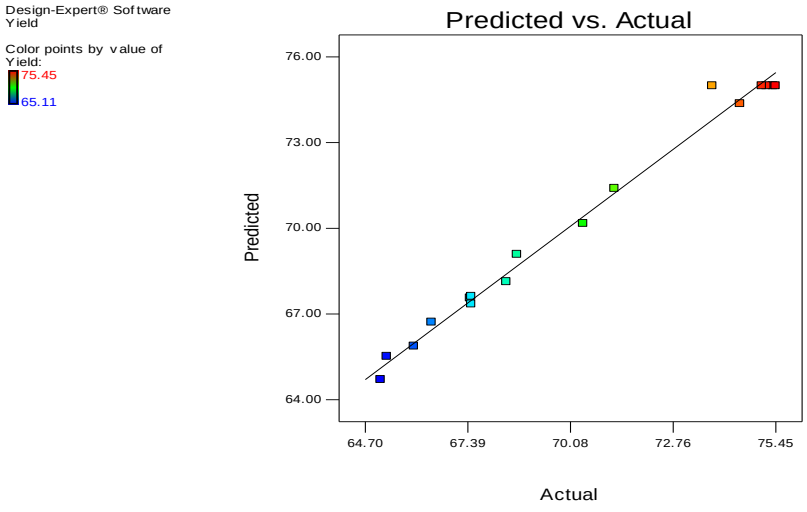

Figure 2. Predicted vs. actual plot of response values

Table 3. ANOVA analysis for response surface quadratic model

Source	Sum of Squares	df	Squares Mean	F value	P value Prob>F	
Model	243.56	9	27.06	73.68	<0.0001	Significant
A	18.42	1	18.42	50.16	0.0002	
B	5.23	1	5.23	14.25	0.0069	
C	3.63	1	3.63	9.89	0.0163	
AB	0.18	1	0.18	0.48	0.5106	
AC	0.032	1	0.032	0.088	0.7751	
BC	29.11	1	29.11	79.25	<0.0001	
A^2	121.11	1	121.11	329.77	<0.0001	
B^2	16.69	1	16.19	45.43	0.0003	
C^2	33.38	1	33.38	90.89	<0.0001	
Residual	2.57	7	0.37			
Lack of Fit	0.71	3	0.24	0.51	0.6979	Not significant
Pure Error	1.86	4	0.47			
Cor Total	246.13	16				

Where: A- Sodium sulfide concentration, B- extraction time and C- mixing ratio

The coefficient term significance is determined by the F-value and p-value. The smaller the value of p and a larger value of F, the more significant is the model. The F-value of 73.68 from the model suggested the model was significant. There is only a 0.10% chance that a "Model F-value" this large might happen because of the noise. Values of "Prob > F" below 0.0500 show that the term of the models are important. In this case, A, B, C, BC, A^2, B^2 and C^2 terms are significant. Lack-of-fit is not significant which represents the model fits well and there is a substantial effect on factors of the output response (Table 3).

3.3 Interaction study

Individual and cumulative effects, as well as the joint interactions between the parameters on the dependent variables, were described using contour plots and response surfaces. In this study, the contour plots and response surface were described by the regression model for BBD which was developed using Design-expert 7.0.0 software. The interaction of concentration of reducing agent (Na_2S) and time of extraction has a progressive effect on the obtained yield of keratin as shown in Figures 3 (a) and (b). It was shown that the keratin yield was increased to 73.79% as Na_2S from 0.2M up to 0.4M and then it was declined when the Na_2S concentrations were further increased. Comparing the lower and the upper limit of the extraction time at a different Na_2S concentration, the keratin with an extraction time of 6 h had a more positive effect on keratin yield than with an extraction time of 4 h. Figures 4 (a) and (b) showed the interaction between concentration of

reducing agent (Na_2S) ($X1$) and mixing ratio ($X2$) with respect to the yield of keratin. Increasing the concentration of reducing agent from 0.325 M to 0.55 M with mixing ratio from 22.25 to 30 g/l enhanced the mass of keratin gained. However, at below 0.325 M and above 0.55 M, a gradual decrease in the response was observed. The interaction effect of extraction time and the mixing ratio was shown in Figures 5 (a) and (b). At the lower limit of the extraction time, the lower value of the mixing ratio (20 g/l) has a high yield of keratin (71.392%) than the higher limit. However, as the extraction time increased a high yield of keratin (74.357%) was obtained at the upper limit of the mixing ratio (30 g/l). Finally, the keratin yield was constant after mixing a ratio of 30 g/l and around the extraction time of 5.75 h.

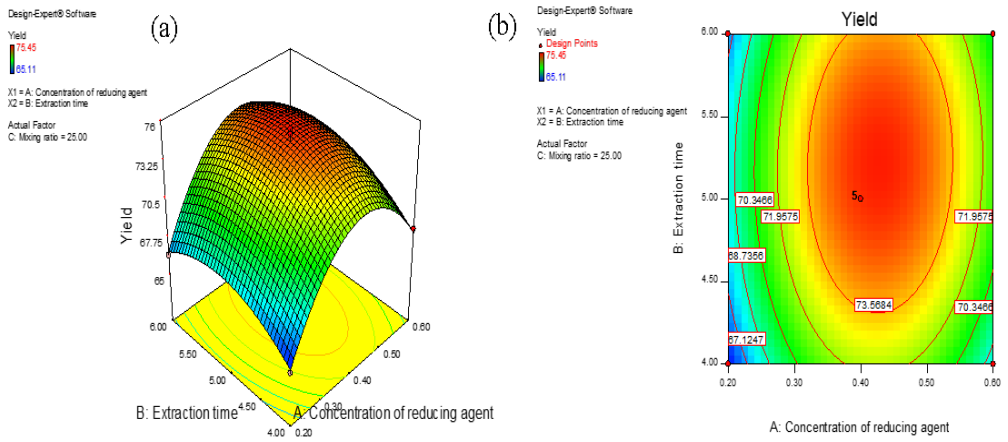

Figure 3. Contour plot (a) and 3D response surface (b) showing the interaction effect of reducing agent and extraction time on the keratin yield

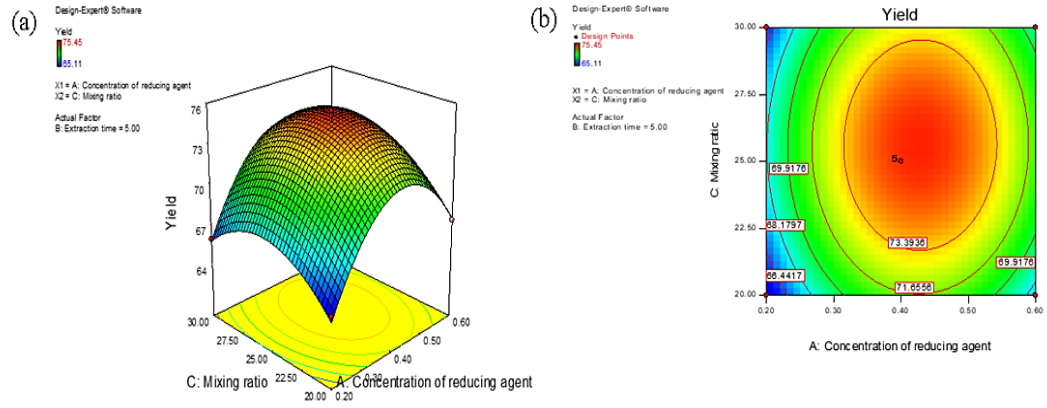

Figure 4. Contour plot (a) and 3D response surface (b) showing the interaction effect of reducing agent and mixing ratio on the keratin yield

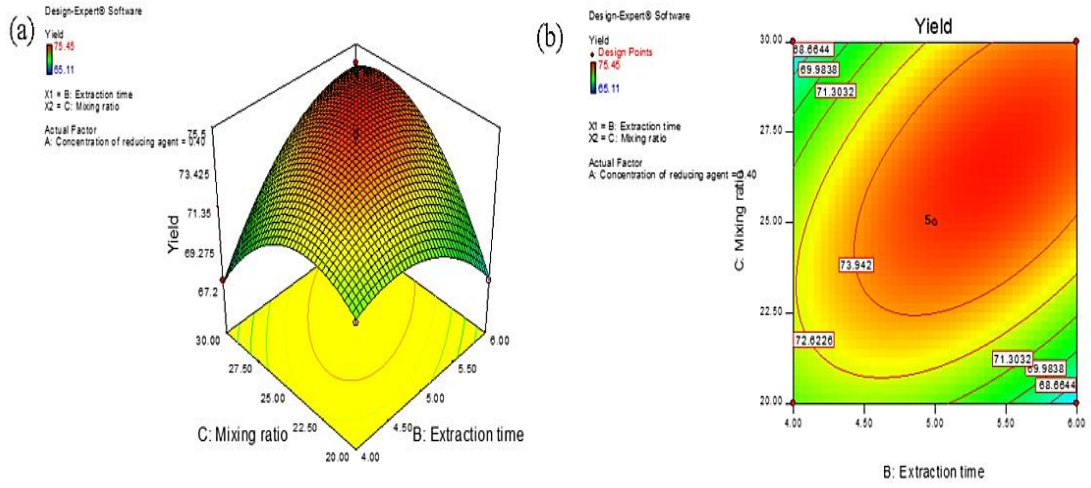

Figure 5. Contour plot (a) and 3D response surface (b) showing the interaction effect of reducing agent and extraction time on the keratin yield

3.4 Optimization of the model and process parameters

The criterion of optimization for the choice of optimal functioning conditions using the quadratic BBD based model was to get the maximum keratin yield with the constrained process factors to the experimental values. Numerical optimization, graphical optimization, and point prediction are ways of expressing optimum process condition and its result. However, all of this optimization method gives similar optimized values for process parameters and the results. A maximum keratin yield of 75.3872% was obtained with the desirability of 0.994 at 0.43M, 5.44 h and 26.65 g/l of Na_2S, extraction time and mixing ratio, respectively.

After optimization, triplicate experiments were performed using the predicted optimized conditions. At these conditions, the mean percentage of keratin yield (74.96%) was obtained. Thus, these results are in correspondence with the predicted values, and hence reflected the applicability of RSM.

3.5 Characterization of keratin protein

3.5.1 UV Visible spectroscopy

The absorption spectra on the solution of keratin exhibited a wide peak in the range of 200-280 nm (Figure 6). UV-Vis absorption measurement of extracted keratin solution showed initial peak wavelength at 220 nm related by the amino acids and carboxylic acid groups forming peptide bond, and the maximum peak at 280 nm caused by the aromatic ring portion of amino acids groups [19]. In general, the fluorescence of keratin was mainly due to tryptophan and tyrosine residues. Keratin is absorbed predominantly in the far UV, but had an absorption extension as far

as 400 nm. The main chromospheres absorbing in the UV region are aromatic compounds of amino acids such as tryptophan, phenylalanine and tyrosine which are existent in the keratin series [20].

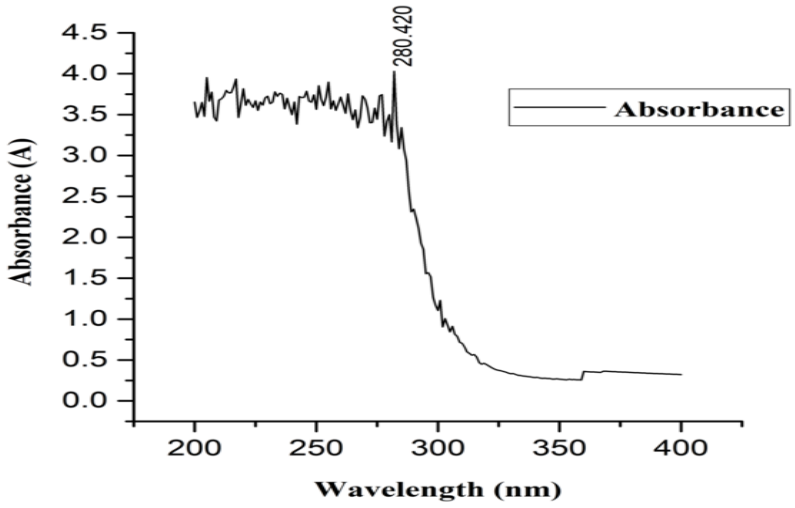

Figure 6. UV visible spectrum of extracted keratin

3.5.2 Fourier transform infrared spectroscopy (FTIR) analysis

The FTIR spectrum obtained for three keratin samples matched the typical spectrum of keratinous protein samples from feathers (Figure 7). All three protein samples show almost similar characteristic peaks. The FTIR characterization of keratin extracts expected to have characteristic peaks corresponding to important functional groups like -CO-NH-, $-NH_2$, -CNH, -C-H. The relatively broad peak in the region of 3300 cm^{-1} corresponds to hydrogen-bonded -N-H and -O-H stretching motion out of amide functionality and absorbed water. The less intense peak in the region of 2900-3100 cm^{-1} signifies -C-H and -N-H groups stretching vibrations. The carbonyl group of amide functionality occurs in the region of 1600-1700 cm^{-1}. An observed peak at 1630 cm^{-1} is assigned to amide carbonyl (-C=O) functional group stretching vibration. The observed peak at 1230 cm^{-1} corresponds to -CNH group comprising -C-N- and -C-C- groups stretching vibrations and -N-H group bending vibration. The bending vibration of $-CH_2$ group occurred at 1450 cm^{-1} [21]. The intense sharp peak at 1525 cm^{-1} corresponds to -C-N-H group bending vibration [22]. The medium intense sharp peaks at 1100 and 920 cm^{-1} correspond to -C-N- group stretching vibration. These FTIR results particularly show the presence of characteristic peaks like amide -N-H, -C=O, -C-N- and -CNH functionalities which confirm building block amino acids forming peptide groups of keratin protein.

3.5.3 Scanning electron microscope (SEM) analysis

The morphology of keratin was observed by SEM with a coated carbon conductive tape in a model FEI, INSPCT-F50, Germany. As shown in Figure 8, the powder keratin was a smooth surface with heterogeneous granulate and texture was merged and embedded. This nature of morphology may be due to the wider poly dispersity of keratin protein. The SEM image of keratin fibers showed a round cross-section and possessed many micropores. This nature of microstructure could be

formed as severe double diffusion keratin filament and small particles in dust form or coagulation bath [18].

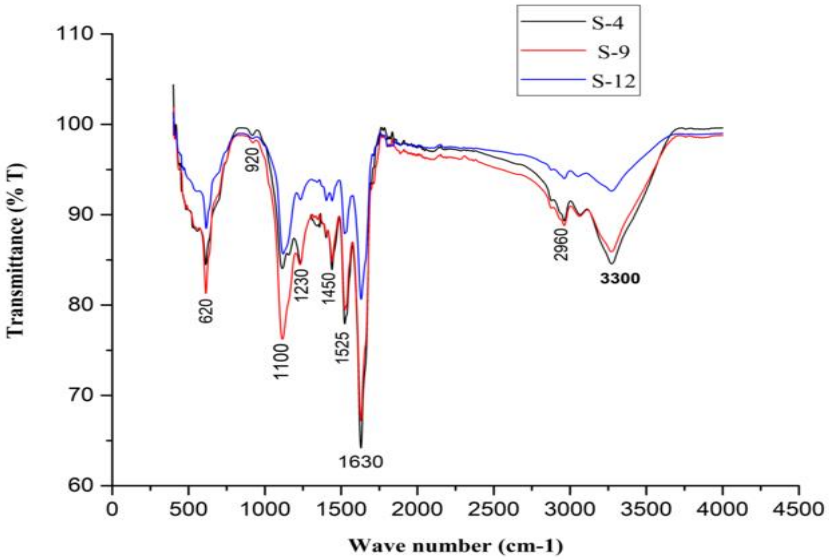

Figure 7. FTIR spectra profile of powdered keratin

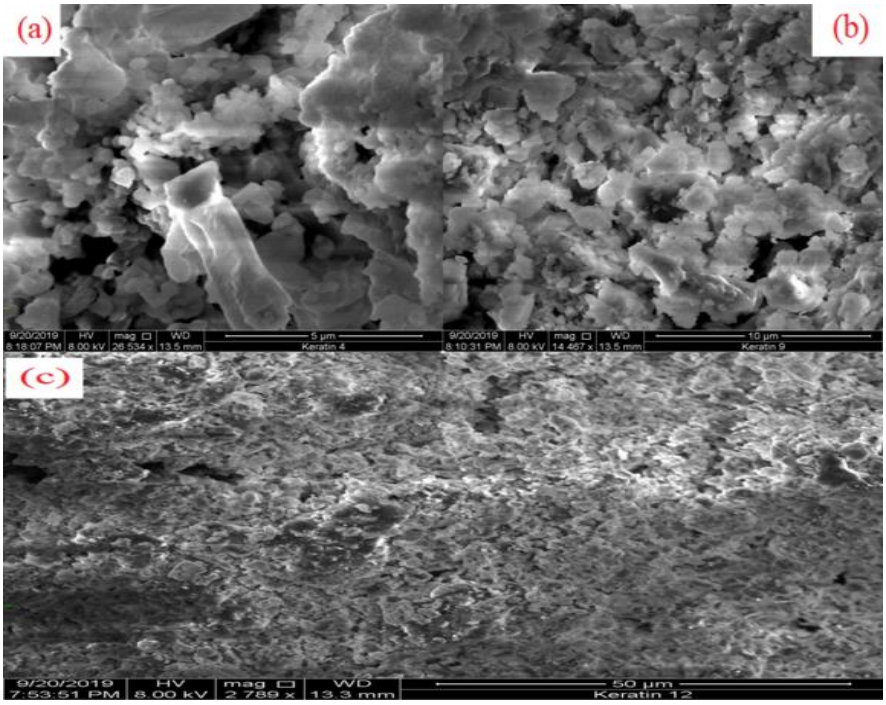

Figure 8. SEM images of keratin:
(a) 26,534× magnification, (b) 14,467× magnification and (c) 2,789 × magnification

3.5.4 X-ray diffraction (XRD) analysis

From the extracted powder keratin, the diffraction angle 2(ɵ) in different peak showed at 5.04º, 9.4º, 11.5º, 17.5º 18.8º, 21.8º and 38.2º (Figure 9). The peak showed in the range of 17.5º to 21.8º represented a protein molecule forming β- sheet structure [5]. The XRD spectra of powder keratin have a slight shoulder or small peak indicating a small ȧ-helix at around 9.4º and most of crystalline characteristics of the keratin have strong intermolecular and intramolecular interaction because of hydrogen bonding [13]. The obtained data showed the effect of the solvent and reducing agent on the crystallization of keratin, and confirmed the tendency of keratin forming β-sheet structure form and sodium sulfide cast form.

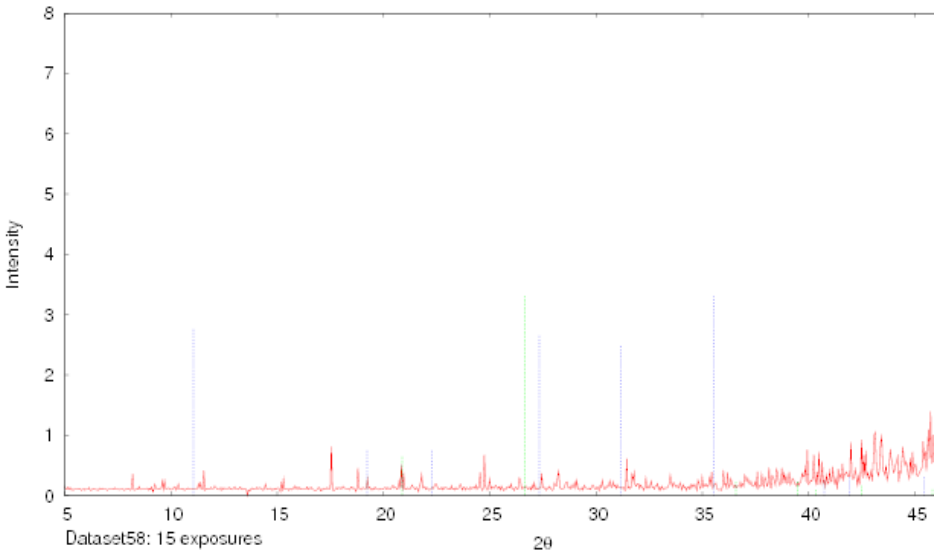

Figure 9. X-ray diffraction of powder keratin

4. Conclusions

Keratin was successfully extracted from chicken feather by using Na_2S as a reducing agent and chemical reduction method. Feathers soaked in diethyl ether, solubilized by sodium sulfide and precipitated with ammonium sulfate and partially purified by NaOH were the most necessary steps to extract keratin. The keratin extraction from the chicken feather waste was optimized for three constraints: reducing agent concentration, extraction time and mixing ratio. The experimental design was performed using the response surface methodology tool and three factors with three levels were used with a total of 17 experiments. The optimum parameters for extraction of keratin were found at reducing agent concentration of 0.43 M, extraction time at 5.53 h and mixing ratio of 26.65 g/l with a keratin yield 75.39% at 0.983 desirabilities using Box- Behnken method. All three parameters had a positive effect on the keratin yield, and from the interaction effects, the extraction time and mixing ratio had a higher significant effect on keratin yield than others. The result of sample characterization showed that non-keratin components were removed through the extraction process. Existence of the fundamental chemical compositions and physical characteristics of keratin was observed using UV-visible spectroscopy, Fourier Transform Infrared Radiation (FTIR) spectroscopy, Scanning Electron Microscopy (SEM) and X-ray Diffraction (XRD) analyses.

References

[1] Esparza, Y., Ullah, A., Boluk, Y. and Wu, J., 2017. Preparation and characterization of thermally crosslinked poly(vinyl alcohol)/feather keratin nanofiber scaffolds. *Matererials and Design*, 135 (5), 1-9.

[2] Reichl, S., Borrelli, M. and Geerling, G., 2011. Keratin films for ocular surface reconstruction. *Biomaterials*, 32, 3375-3386.

[3] Tesfaye, T., Sithole, B. and Ramjugernath, D., 2017. Valorisation of chicken feathers: a review on recycling and recovery route-current status and future prospects. *Clean Technologies and Environmental Policy*, 19 (10), 2363-2378.

[4] Reddy, N. and Yang, Y., 2007. Structure and properties of chicken feather barbs as natural protein fibers. *Journal of Polymer and the Enviroment*, 15, 81-87.

[5] Aluigi, A., Zoccola, M., Vineis, C., Tonin, C., Ferrero, F. and Canetti, M., 2007. Study on the structure and properties of wool keratin regenerated from formic acid. *International Journal of Biological Macromolecules*, 41 (3), 266-273.

[6] Vineis, C., Varesano, A., Varchi, G. and Aluigi, A. 2019. Extraction and characterization of keratin from different biomasses. In: Sharma, S., Kumar, A., eds. *Keratin as a Protein Biopolymer*. Cham: Springer International Publishing, 35-76.

[7] Gupta, A., Perumal, R., Yunus, R.B.M. and Kamarudin, N.B., 2012. Extraction of keratin protein from chicken feather. 8 (6) 732–737. [online] Available at: https://pdfs.semanticscholar.org/ c72d/3cb1ded22997f6aa85ded41f8f1180d9d1aa.pdf

[8] Sharma, S. and Gupta, A. 2016. Sustainable management of keratin waste biomass: applications and future perspectives. *Brazilian Archives of Biology and Technology*, 59: e16150684, http://dx.doi.org/10.1590/1678-4324-2016150684.

[9] Rouse, J.G. and Van Dyke, M.E., 2010. A Review of keratin-based biomaterials for biomedical applications. *Materials*, 3 (2), 999-1014.

[10] Sharma, S., Gupta, A., Chik, S. M.S.T., Kee, C.Y.G., Podder, P.K., Thraisingam, J. and Subramanian, M., 2016. Extraction and characterization of keratin from chicken feather waste biomass : a study. *The National Conference for Postgraduate Research*, Universiti Malaysia Pahang, December, 2016, 693-699.

[11] Yadav, R.N., 2017. A hybrid approach of Taguchi-Response Surface methodology for modeling and optimization of Duplex Turning process. *Measurement*, 100, 131-138.

[12] Eslahi, N., Dadashian, F. and Nejad, N.H., 2013. An investigation on keratin extraction from wool and feather waste by enzymatic hydrolysis. *Prepative Biochemistry and Biotechnology*, 43 (7), 624-648.

[13] Sharma, S., Gupta, A., Kumar, A., Kee, C.G., Kamyab, H. and Saufi, S.M., 2018. An efficient conversion of waste feather keratin into eco-friendly bioplastic film. *Clean Technologies and Environmental Policy*, 20 (10) 2157-2167.

[14] Wingfield, P., 1998. Protein precipitation using ammonium sulfate. *Current Protocols in Protein Science*, Hoboken, NJ, USA: John Wiley & Sons, Inc., A.3F.1-A.3F.8.

[15] Shah, A., Tyagi, S., Bharagava, R.N. and Belhaj, D., 2019. *Keratin as a Protein Biopolymer*. Cham: Springer International Publishing..

[16] Goddard, D. R. and Michaelis, L. 1934. A studies on keratin. *The Journal of biological chemistry*, 106 (2) 605–614.

[17] Dou, Y., Zhang, B., He, M., Yin, G. and Cui, Y., 2016. The structure, tensile properties and water resistance of hydrolyzed feather keratin-based bioplastics. *Chinese Journal of Chemical Engineering*, 24 (3), 415-420.

[18] Kamarudin, N.B., Sharma, S., Gupta, A., Kee, C.G., Chik, S.M.S.B.T. and Gupta, R., 2017. Statistical investigation of extraction parameters of keratin from chicken feather

using Design-Expert. *3 Biotech*, 7 (2), 127, https://doi.org/10.1007/5/3205-017-0767-9.

[19] Idris, A., Vijayaraghavan, R., Rana, U.A., Patti, A.F. and MacFarlane, D.R., 2014. Dissolution and regeneration of wool keratin in ionic liquids. *Green Chemistry*, 16 (5), 2857-2864.

[20] Yamauchi, K., Yamauchi, A., Kusunoki, T., Kohda, A. and Konishi, Y., 1996. Preparation of stable aqueous solution of keratins, and physiochemical and biodegradational properties of films. *Journal of Biomedical Materials Research*, 31 (4), 439-444.

[21] Wojciechowska, E., Włochowicz, A. and Wesełucha-Birczyńska, A., 1999. Application of fourier-transform infrared and raman spectroscopy to study degradation of the wool fiber keratin. *Journal of Molecular Structture*, 511-512, 307-318.

[22] Jose, S., Nachimuthu, S., Das, S. and Kumar, A., 2018. Moth proofing of wool fabric using nano kaolinite. *Journal of Textitle Institute*, 109 (2), 225-231.