ORIGINAL PAPER

Straightforward and Highly-Efficient Feather Keratin Extraction by Systematic Optimization of Sodium Sulfide Treatment Process

Gholamreza Goudarzi[1], Fatemeh Dadashian[1*], Alireza Vatanara[2], and Zargham Sepehrizadeh[3]

Abstract- **It is highly economical to extract keratin from the waste chicken feathers due to their high keratin content, plentiful availability, and sustainable resources. Various methods of keratin extraction have been reported in the last few decades. In addition, sodium sulfide (Na_2S) treatment has received more attention due to its simplicity and ability to produce on an industrial scale. Although several studies have been conducted on improving keratin extraction yield through Na_2S treatment, there need to be more systematic studies to evaluate and optimize the effect of different extraction parameters and their interactions to maximize extraction efficiency. In this research, the response surface method (RSM) established on the central composite practical design (CCPD) was employed satisfactorily to understand the influence of experimental parameters and their interactions to determine the optimal conditions for keratin extraction. Na_2S concentration, extraction time, and extraction temperature were chosen as the most critical parameters for investigation. Experimentally, the extraction yield of 94±0.5% was obtained under the RSM-optimized conditions (i.e., 80 °C, 6.3 h, and 32.0 g.l^{-1} Na_2S concentration), which is in close agreement with the model-predicted value (95%). The optimized keratin extraction yield in this study is relatively high. Physicochemical properties of the extracted powder were characterized by** sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM). The results revealed that the extracted material contained both β-keratins and α-keratins, which have great potential for advanced healthcare and medical applications.

Keywords: chicken feather, keratin, sodium sulfide treatment, response surface methodology

G. Goudarzi and F. Dadashian,
Department of Textile Engineering, Amirkabir University of Technology, Tehran, Iran.

A. Vatanara
Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

Z. Sepehrizadeh
Department of Biotechnology and Biotechnology Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

Correspondence should be addresses to F. Dadashian
e-mail: dadashia@aut.ac.ir

I. INTRODUCTION

A major fibrous protein in nature is keratin which can be found in feathers, hair, nails, wool, horns, and hooves [1-4]. Keratin has remarkable characteristics. It is a biocompatible and biodegradable polymer that supports cellular adhesion and proliferation. These properties, along with abundance, make it valuable for developing keratin-based materials in the form of sponges, films, and scaffolds for diverse advanced applications [4-9], such as regenerative medicine [10-12], wound healing [13-16] and drug delivery [6,17-19].

Because raw poultry feathers are composed of over 90% keratin with inexpensive and sustainable sources, they are known to be the most abundant keratin source [20-22]. In general, keratin in chicken feathers has high stiffness and insolubility in various aqueous and organic media such as water, weak acids, and bases, as well as in non-polar solvents due to a high degree of disulfide cross-linkages, hydrogen bonds, salt bonds, and hydrophobic interactions within polypeptide chains and structural features, like crystallinity [1,23,24]. The main molecules of keratin are polypeptide chains. These chains can be curled in the form of a helix (α-helix conformation) or side-by-side chains connected in the form of folded sheets

(β-sheets conformation) [25]. The β-keratins are harder than α-keratins. Keratins are based on β-forms in the hard corneal materials of reptiles and birds such as scales, claws, beaks, and feathers [25,26]. Mammalian keratins are coiled-coil α-helix rather than β-sheets [27]. As a structural protein, the keratin of feathers contains complicated structures of α-helix and β-sheet crystallites and is highly crosslinked because of 7% mol cysteine [28]. With these characteristics, keratin extraction is not an easy process. Different methods have been reported for the extraction of keratin from chicken feathers which in general can be classified into chemical methods [6], enzymatic and microbial methods [29,30], steam explosion, supercritical water [31,32], and microwave irradiation [33,34]. The most conventional chemical methods for keratin extraction are based on oxidation and reduction reactions [35]. Extracting keratin with ionic liquids is another considerable chemical method [36].
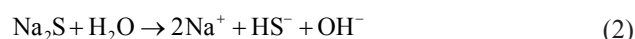
During the process of keratin extraction by reducing agents, such as 2-mercaptoethanol and dithiothreitol [37], the reduction of cystine to cysteine molecules leads to the breakdown of the disulfide bonds, as shown in Eq. (1) [38]:

$$2R-CH_2-SH \underset{+2H}{\overset{-2H}{\rightleftharpoons}} R-CH_2-S-S-CH_2-R \qquad (1)$$
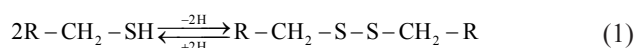
To improve keratin extraction yield, other chemical compounds like surfactants, urea [39,40], and thiourea [41] were incorporated into the reduction medium. By disrupting hydrogen bonding between protein chains, urea facilitates access to disulfide bonds for reducing agents. For example, Schrooyen *et al.* reported 75 and 20% values for keratin extraction yields with and without adding urea to the 2-mercaptoethanol solution, respectively, which indicates the influence of the presence of such compounds on the extraction yield [42]. Also, the detergent sodium dodecyl sulfate (SDS) accelerates the extraction process by preventing protein chain aggregation. However, it does not affect the amount of extraction yield [43,44], although Yamauchi *et al.* reported that SDS improved the extraction yield [39]. It has also been claimed that the SDS surfactant forms a complex with denatured keratin to provide a stable solution of keratin by preventing re-crosslinking during the dialyzing process, which removes the reducing agent from the extracted solution [43]. However, interference of detergents in keratin solution with the chemical and physical analyses is an issue, and it is hard to completely remove it due to the formation of stable complexes with keratin [41,45]. In general, combining several reagents in an extraction solution makes no consistent protein yields, and protein hydrolysis may occur [41].

Sodium sulfide treatment, compared with other reducing agents, especially 2-ercaptoethanol, as the most conventional reducing agent for keratin extraction, is a more simple and cost-effective method so that it can be industrially feasible in the extraction of keratin [44,45]. Moreover, it has been reported that sodium sulfide treatment does not cause significant damage to the keratin protein chains [43,46]. Sodium sulfide in water dissociates into hydrosulfide and hydroxyl ions (Eq. (2)):

$$Na_2S+H_2O \rightarrow 2Na^+ + HS^- + OH^- \qquad (2)$$

With these two anions, sodium sulfite reduces and cleaves the keratin disulfide bonds. In addition, the alkaline conditions governing this process can facilitate keratin extraction due to rupturing the hydrogen bonds and loosening the structure of keratin materials [47,48]. The efficiency of keratin extraction with Na_2S can be high without using auxiliaries like urea and sodium dodecyl sulfate or mixing of reducing agents [23].

The present research was carried out to obtain yield optimization of keratin extraction by sodium sulfide processing. The optimization studies have been performed using response surface methodology (RSM). RSM is a statistical method to optimize process conditions. It can determine the influence of different parameters and their interactions on the factors under study (responses) and provide the regression model equation and the operating conditions. There have been previous attempts to use the RSM to optimize the extraction of keratin from chicken feathers with sodium sulfide [49,50], but these efforts varied in the number and kind of variables they used and the optimum yields they achieved. According to earlier research and preliminary experiments, the best and minimum independent parameters have been selected in this study to achieve the highest efficiency with RSM. Besides, optimized extracted keratin was characterized by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) analysis, SDS-PAGE and scanning electron microscopy (SEM).

II. EXPERIMENTAL

A. Materials and Processing

Chicken feathers were obtained fresh from a local chicken slaughterhouse (in the southeast of Tehran, Iran). Sodium sulfide and hydrochloric acid were obtained from Sigma-Aldrich (USA). Chicken feathers were washed with warm water and then scoured with a nonionic detergent (Irgasol NA was provided by Ciba Co.) at 60 °C for 1 h with agitation and thoroughly rinsed with water. After that, to entirely remove greasy matter, the cleaned feathers were immersed in ethanol for 24 h and then rinsed. Finally, the

TABLE I
INDEPENDENT VARIABLES AND LEVELS USED FOR THE CENTRAL COMPOSITE PRACTICAL DESIGN (CCPD)

Variables	Symbol code	Levels				
		-1.316	-1	0	+1	+1.316
Temperature (°C)	A	30	36	55	74	80
Time (h)	B	0.5	1.9	6.3	10.6	12
Na$_2$S conc. (g.l^{-1})	C	5	11.6	32.5	53.4	60

wet feathers were dried at room temperature.

B. Experimental Design of the Keratin Extraction Process

A central composite practical design (CCPD) from RSM was operated to analyze the effect of three extraction variables (sodium sulfide concentration, time, and temperature) on the yield of extracted keratin from chicken feathers at three levels. The liquor to goods ratio, namely the "mass of feather/volume of solution", in all the extractions was limited to 1:20. For each variable, three levels were assigned in codes -1, 0, and +1. The minimum and maximum levels of code were shown by -1.316 and +1.316, respectively. Table I shows the values of independent variables with their codes in five levels produced by the Design Expert software (Version 11.1.0.1, 2018; Stat-Ease Inc., Minneapolis, MN, USA). With three independent variables (k=3), the total experiment calculated from 2k+2k+6 was 20. Five replications were accomplished at the center point to define the pure error. Based on the preliminary study, the levels of the variables were defined.

C. Sodium Sulfide Extraction

One gram of the clean dried feathers was cut into small pieces on the centimeter scale, placed in a glass container with a cap, and immersed in Na$_2$S solution. For complete immersion of feathers in solution, the liquor ratio (mass of feather to solution ratio) was considered 1:20. The container cap was closed tightly and put in an incubator with shaking (120 rpm). Temperature, time, and Na$_2$S concentration were adjusted according to the experimental design (Table II). At the end of processing, the extraction solution was centrifuged at 10000 xg at 4 °C for 15 min. The supernatant that contained dissolved keratin was decanted from feather residuals. By drying feather residues, the extraction yield was evaluated. The extracted yield was calculated as given in Eq. (3):

$$\text{Extraction yield } (\%) = (W - W')/W \times 100 \qquad (3)$$

where W′ is the weight of residual feathers after the

extraction process and W is the initial weight of the feather sample after extraction.

C.1. Protein Precipitation

The supernatant was placed in a beaker and stirred. Hydrochloride acid (0.5 N) was added slowly dropwise. The keratin content in the supernatant was precipitated at its isoelectric point (pH 3.5). The precipitates were collected by centrifugation at 10000 xg for 15 min, then washed with distilled water three times, and oven-dried. The keratin powder was obtained by grinding dried keratin sediment.

D. Characterization Techniques

D.1. Protein Measurement

Protein concentration in the supernatant was defined by a spectrophotometer at 280 nm using bovine serum albumin (BSA) as a standard.

D.2. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used according to the method of Laemmli [46] to estimate the molecular weight of extracted keratin. One mg of keratin samples was dissolved in 100 μL of a buffer solution (62.5 mM Tris–HCl, pH 6.8), 2% SDS, 10 w/v% glycerol, 0.01% bromophenol blue containing 5% of 2-β-mercaptoethanol and heated at boiling for 10 min. The protein separation was carried out on a polyacrylamide gel consisting of 5% stacking gel and 15% separating gel. The stacking and the separating gels were 5 and 15% polyacrylamide, respectively. The electrophoresis was run at 80 V for 30 min, pursued by 120 V for 60 min. The gel was washed with distilled water and with a staining solution (Coomassie brilliant blue R-250) stained. The samples were destained with dilute methanol and glacial acetic acid solution in deionized water. A molecular weight protein ladder (SinaClon, Iran) was used for calibration.

D.3. Fourier-Transform Infrared Spectroscopy (FTIR)

The chemical structures analysis of keratin powders was performed in a Nicolet is 10 FTIR Spectrometer (USA).

The spectra were recorded in the range of 4000 cm^{-1} to 400 cm^{-1} with transmission mode and collected with 64 scans/min and 4 cm^{-1} resolution. The analysis was performed in triplicate.

D.4. X-Ray Diffraction
The crystal structure analysis was performed using an X-ray diffractometer (INEL-Equinox-3000, France) with Cu Kα radiation worked at 40 kV, 30 mA. Data for qualitative analysis were recorded in the angle range of 5°≤2Θ ≤90 at the rate of 0.03°/min.

D.5. SEM
The morphology and surface topography of the keratin powder were evaluated by scanning electron microscopy (SEM; Zeiss Supra VP 40, Germany).

D.6. Statistical Analysis
The derived equation from software that best expresses the relationship of independent variables and their interaction with the keratin yield% (response) was a quartic polynomial equation. For a detailed evaluation of the produced model, analysis of variance (ANOVA) was run. Also, the coefficient of determination (R^2) specified the quality of the fitted model.

III. RESULTS AND DISCUSSION
A. Results of RSM
For this design, according to the preliminary works and the previous studies [44,49], time, temperature, and concentration were selected as effective and independent parameters in the extraction of keratin by sodium sulfide. In previous research [49], five variables have been utilized, some of which were not independent variables or in another research [50], one of the important parameters, i.e. temperature, was not taken into account. Preliminary experiments were conducted to decide the influence of sodium sulfide concentration on the keratin extraction and define the range of sodium sulfide concentration used in the RSM design. The protein concentration of the supernatant increased with a higher Na$_2$S concentration.

A.1. Model Fitting
Table II shows the influence of temperature, time, and Na$_2$S concentration, determined using multiple regression, on the yield of keratin extraction from feathers from 20 steps produced by RSM. Table III summarizes the ANOVA outputs of the model.

The correlation between the keratin yield and the extraction variables within the studied ranges was well described by a quartic polynomial model with a model

TABLE II
EXPERIMENTAL VARIABLES (A TEMPERATURE, B TIME, AND C NA$_2$S CONCENTRATION) AND RESPONSES

	Coded level			Actual level of variables			
Run	A	B	C	A (°C)	B (h)	C (g.l^{-1})	Keratin yield (%)
1	1.000	1.000	1.000	74.0	10.6	53.40	95
2	−1.316	0.000	0.000	30.0	6.3	32.50	80
3	1.000	−1.000	−1.000	74.0	1.9	11.60	82
4	−1.000	−1.000	−1.000	36.0	1.9	11.60	74
5	−1.000	1.000	1.000	36.0	10.6	53.40	86
6	−1.000	−1.000	1.000	36.0	1.9	53.40	78
7	0.000	0.000	0.000	55.0	6.3	32.50	87
8	0.000	1.316	0.000	55.0	12.0	32.50	91
9	0.000	0.000	0.000	55.0	6.3	32.50	87
10	1.000	1.000	−1.000	74.0	10.6	11.60	88
11	0.000	−1.316	0.000	55.0	0.5	32.50	75
12	1.000	−1.000	1.000	74.0	1.9	53.40	90
13	0.000	0.000	−1.316	55.0	6.3	5.00	12
14	−1.000	1.000	−1.000	36.0	10.6	11.60	78
15	0.000	0.000	1.316	55.0	6.3	60.00	84
16	1.316	0.000	0.000	80.0	6.3	32.50	95
17	0.000	0.000	0.000	55.0	6.3	32.50	87
18	0.000	0.000	0.000	55.0	6.3	32.50	88
19	0.000	0.000	0.000	55.0	6.3	32.50	87
20	0.000	0.000	0.000	55.0	6.3	32.50	87

P-value<0.0001 and a very high coefficient of determination (R^2=0.9998) and high adjusted R^2 =0.9994. The lack of fit result was not significant at the 5% level (p>0.05), meaning the model fit the data very well (the P-value for the model was 0.3632).

The regression equation of the quartic model in terms of coded variables to predict keratin yield (Y) was considered as follows (Eq. (4)):

$$Y = 87.25 + 5.70A + 6.08B + 27.35C - 0.1250AB + 0.3750AC + 0.375BC - 2.45B^2 - 22.66C^2 - 0.625ABC - 3.2A^2B - 23.98A^2C - 0.8238AB^2 + 21.74A^2B^2 \qquad (4)$$

where A is temperature, B is time, and C is sodium sulfide concentration. The coefficients of each term in the regression equation show the effect of any parameter (A, B, and C) and the interaction between parameters. The negative sign in the regression equation indicates an antagonistic effect, and the positive sign indicates a synergistic effect. In this fitting obtained model for the response variable, the temperature (p<0.001), the time (p<0.001), and Na_2S concentration (p<0.001) affected the keratin yield linearly.

B. Effects of Temperature, Time, and Sodium Concentration on the Levels of the Keratin Yield

Fig. 1 shows the effects of process factors on the keratin extraction yield at -1, 0, and +1 levels. The response surface plots of the quartic model were complex. At all time levels (-1, 0, +1), increasing Na_2S concentration enhanced the keratin yield (Fig. 1a). Increasing the temperature to moderate temperature improved the yield. After that, the yield fell at a higher temperature. The yield was greater at higher and lower temperatures. At moderate temperatures and lower concentrations, the yield was lower. The pattern of -1 and +1 levels of temperature was similar; the yield was higher at +1 level. At low and high times, the yield was greater. As the concentration increased, the yield first increased and then decreased. At the moderate temperature (0 level), the yield increased with higher concentrations, and time had no significant effect. At lower Na_2S concentration (-1 level), time did not significantly affect keratin yield extraction. With higher and lower temperatures, at longer and shorter times, the yield was greater. At moderate temperatures, it had the lowest yield.

TABLE III
ANALYSIS OF VARIANCE (ANOVA) FOR THE FITTED QUARTIC MODEL FOR KERATIN YIELD

Source	Sum of squares	d_f	Mean square	F-value	P-value*
Model	5743.95	13	441.84	2651.05	<0.0001
A-temperature	112.5	1	112.5	675	<0.0001
B-time	128	1	128	768	<0.0001
C-Na$_2$S conc.	2592	1	2592	15552	<0.0001
AB	0.125	1	0.125	0.75	0.4298
AC	1.13	1	1.13	6.75	0.0408
BC	1.13	1	1.13	6.75	0.0408
B²	28.9	1	28.90	173.4	<0.0001
C²	2464.9	1	2464.90	14789.4	<0.0001
ABC	3.13	1	3.13	18.75	0.0049
A²B	24.81	1	24.81	148.86	<0.0001
A²C	1389.97	1	1389.97	8339.83	<0.0001
AB²	1.64	1	1.64	9.84	0.0201
A²B²	1024.48	1	1024.48	6146.85	<0.0001
Residual	1	6	0.1667		
Lack of fit	0.1667	1	0.1667	1.0000	0.3632
Pure error	0.8333	5	0.1667		
Cor total	5744.95	19			
R²	0.9998				
R² adjusted	0.9994				
Std. dev.	0.4082				
Mean	81.55				
CV (%)	0.5006				

The model F-value of 2461.76 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. *P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, AC, BC, B², C², ABC, A²B, A²C, AB², and A²B² are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

The moderate Na$_2$S concentration (0 level) was similar to the -1 level, but yields at all temperatures were more. At higher Na$_2$S concentrations (+1 level), yields were the greatest at moderate times and temperatures. At moderate times and lower temperatures, the yield was the lowest.

Increasing Na$_2$S concentration raised the yield but with a limit. It is probably due to the number of available disulfide bonds. Earlier research also mentioned this limitation [44]. Regarding the time effect, the greatest extraction belonged to the early hours. The increase in temperature had two opposing effects. The reason could be explained in two aspects. First, sodium sulfide has a high thermal energy storage density due to its high absorption capacity and high absorption heat [51]. As the temperature increases, this heat is absorbed and leading to less extraction. On the other hand, the vapor pressure of the solutes increases at higher temperatures, thereby improving the extraction.

According to the model that the RSM designed, the optimization of the extraction conditions was performed to obtain the highest keratin extraction yield. The optimum extraction conditions were 80 °C, 6.3 h with 32.0 g.l^{-1}. The three repetitions of extraction confirmed the predicted optimal conditions at the obtained optimum conditions. The predicted optimum keratin yield was 95%. The actual

keratin extraction yield was 94±0.5%. The results of this model showed that the experimental data were congruent with the predicted values.

For comparison, in a similar study that used RSM (based on a central composite design (CCD)) to extract keratin from chicken feathers using sodium sulfide as a reducing agent, five parameters were used: the ratio of mass of chicken feather to Na$_2$S (CF:Na$_2$S), extraction time, temperature of reaction, pH and the concentration of Na$_2$S [49]. The yield extraction was reported to be 86.5-91% in a period of 9.5 h at a temperature of 80.9 °C. But in this work, the yield was higher (94%) in a shorter duration time (6.3 h). Moreover, two factors, CF:Na$_2$S and the concentration of Na$_2$S, were not independent variables. In another work, three independent variables (sodium sulfide concentration, extraction time and mixing ratio) were used in RSM (based on the Box Behnken design (BBD)) [50]. The keratin yield was 75.39% at 5.53 h and the effective factor (temperature) was not considered in this design.

C. Scanning Electron Microscopy (SEM)

Fig. 2 shows SEM images of keratin powder surfaces. Hard and stone-like keratin particles are visible in these images with deposited keratin layers (indicated by the circle).
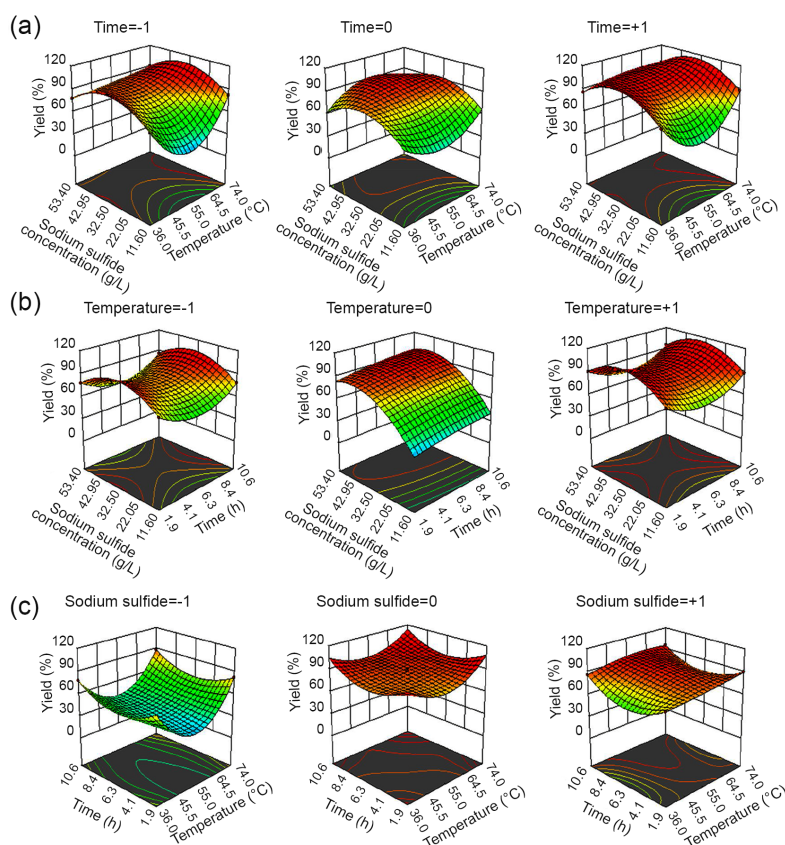

Fig. 1. The three-dimensional response surfaces of keratin yield for the independent factors at -1, 0, +1 levels.
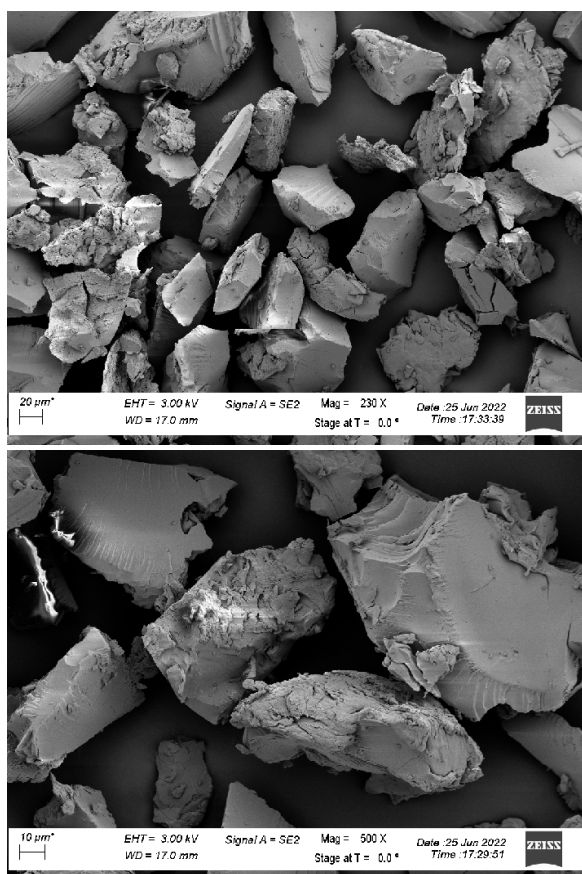
Fig. 2. SEM images of keratin powder in two magnifications. The circle shows the deposited layers.

Regeneration of disulfide bonds between polypeptides (see FTIR of keratin powder in Fig. 4) restored the stiffness of keratin.

D. SDS-PAGE

Fig. 3 displays the molecular weight (kDa) of the regenerated keratin obtained by dissolving chicken feathers using Na_2S under optimal conditions. Lane 1 and 2 contain regenerated keratin and standard protein markers, respectively. Various protein components between 10 and 68 kDa are also observed. Lane 1 shows three bands in the 48-63 kDa range, at about 35, 25, and 20 kDa. Also, keratin with a molecular weight of about 10 kDa can be observed. β-keratins have a smaller molecular mass (10-22 kDa) than α-keratins (40-68 kDa) [26], in extracted keratin powder from chicken feathers by Na_2S, and α-keratins can be seen as shown in the XRD spectrum of a residual feather (Fig. 5). However, the α-structure in reaction with Na_2S is completely extracted and the remains of the β-sheet structure can be seen in the residual feathers. High-sized molecules in the extracted keratin indicate that sodium sulfide does not damage protein backbones as observed in previous studies [47,52].
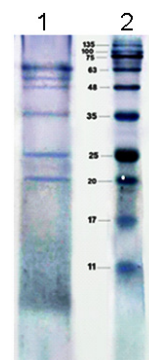

Fig. 3. SDS-PAGE gel of keratin. Lane 2 protein molecular weight standards (kDa), lane 1 the keratin extracted by sodium sulfid

E. Infrared Spectroscopy

The FTIR spectrum of the extracted keratin powder from feathers is presented in Fig. 4. The spectrum shows characteristic absorption peaks of peptide bonds (-CONH-) that are labeled as amide A at 3295 cm^{-1}, amide B at 3075 cm^{-1}, amide I at 1600-1700 cm^{-1}, amide II at 1480-1580 cm^{-1} and amide III at 1220-1300 cm^{-1}. The amide A band and amide B appear from a resonance between the first overtone of amide II and the N-H stretching vibration. The amide A originates mainly from stretching N-H bonds vibration. Amide I was assigned to C=O stretching. Amide II was assigned to N-H in-plane bending and the C–C–N stretching vibrations. Amide III is related to an in-phase combination of C-N stretching and the C=O bending vibrations. Amide I is an excellent band in the protein structure analysis of the secondary structure of proteins [53,54]. The peak at 580 cm^{-1} is the characteristic absorption of the S-S bond [6]. The bands were related to α-helix and β-sheet structures, falling in the range of 1657-1651 and 1631-1621 cm^{-1}, respectively. The absorption peaks in the 1697-1670 cm^{-1} range suggest the formation of disordered structures of keratin [55,56]. The FTIR spectrum of raw feather (Fig. 4) is similar to the FTIR spectrum of extracted keratin and there are no signatures of new functional groups appearing in the regenerated keratin materials [36,57-60].

F. X ray Diffraction

As shown in Fig. 5, the XRD patterns of the untreated chicken feather, residual feather after Na_2S treatment, and the extracted keratin powder were compared to investigate the crystalline variations. It can be seen that both untreated feathers and extracted keratin display two broad characteristic bands at 2θ corresponding to the α-helix and β-sheet structures of the typical β-keratins of the avian feathers [27,61]. The α-helix band was centered at around 9.2° 2θ and the more intensive band assigned to the β-sheet configuration was centered at around 19.6° 2θ [62]. The

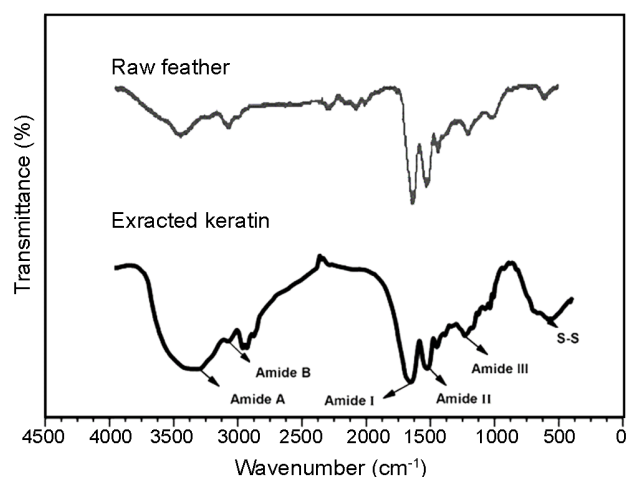Fig. 4. FTIR spectra of the regenerated keratin powder from Na_2S treatment of the chicken feathers under optimized conditions and the raw chicken feather.

diffraction pattern of the residual feather resulting from treatment with Na_2S reaction illustrates the disappearance of the α-helix band, which could be attributed to the destruction of the α-helix structures. Moreover, a broad band at the β-sheet configuration position with a slight peak shift at about 22° and a few other small peaks at higher diffraction angles have appeared. From the results, it could be concluded that the β-sheet crystal has a more tightened structure than the α-helix structure since the availability of the α-helix crystal is more than the β-sheet in reduction by Na_2S [54,63,64]. According to the study on feather residuals, the chicken feather quill is more resistant to decomposition than other parts of the feather (barbs and barbules). Quill is tough, and unlike barbs and barbules, which mainly have the α-structure, it predominantly has
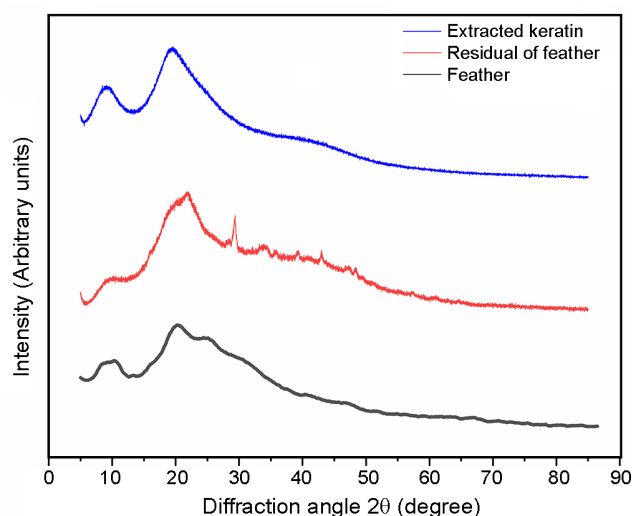

Fig. 5. XRD patterns of natural chicken feathers, residual feathers from treated feathers with Na_2S, and regenerated keratin.

a β-sheet structure [23,65]. Therefore, this β-structure observed in the X-ray of feather residuals could be attributed to residuals of the quill.

On the other hand, the observed peak shift for the β-sheet could be due to more d-spacing alteration in reaction with Na_2S. Furthermore, some new microcrystalline phases could be attributed to the interactions of macromolecules with Na_2S through the reduction reactions, which generated new crystal regions [66]. These minor peaks are indexed to the antiparallel β-sheet structure compared with the natural chicken feather keratin [67].

IV. CONCLUSION

Systematic optimization of the process of extracting keratin from chicken waste feathers with Na_2S by RSM can determine the optimal conditions to obtain maximum keratin yield (about 94%). The optimum conditions for the extraction of keratin were 80 °C, 6.3 h, and 32.0 g.l^{-1} Na_2S concentration. The efficient keratin extraction compared with other methods was higher without using supplements and hazardous materials. In the obtained keratin powder, the XRD and SDS-PAGE results indicate the existence of α- and β-forms. The XRD of residual feathers indicates that α-keratin proteins are more accessible than β-keratins.

Besides, the backbones of proteins are not damaged in optimal conditions. Due to the backbones of proteins that are not damaged in optimal conditions, their applications can be developed. These conditions have made Na_2S treatment an appropriate process for keratin production on the industrial scale from waste feathers as an excellent sustainable source for keratin production on the industrial scale.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

[1] M.A. Khosa and A. Ullah, "A sustainable role of keratin biopolymer in green chemistry: a review", *J. Food Process. Beverages*, vol. 1, no. 1, pp. 8, 2013.

[2] O.D. Fagbemi, B. Sithole, and T. Tesfaye, "Optimization of keratin protein extraction from waste chicken feathers using hybrid pre-treatment techniques", *Sustain. Chem. Pharm.*, vol. 17, pp. 100267, 2020.

[3] D.A. Ertek, N.O. Sanli, Y.Z. Menceloglu, and S.A. Seven, "Environmentally friendly, antibacterial materials from recycled keratin incorporated electrospun PLA films with tunable properties", *Eur. Polym. J.*, vol. 185, pp. 111804, 2023.

[4] M. Zubair, I. Zahara, M.S. Roopesh, and A. Ullah, "Chemically cross-linked keratin and nanochitosan

based sorbents for heavy metals remediation", *Int. J. Biol. Macromol.*, vol. 241, pp. 124446, 2023.

[5] J.R. Richter, R.C. dE Guzman, O.K. Greengauz-Roberts, and M.V. Dyke, "Structure-property relationships of meta-kerateine biomaterials derived from human hair", *Acta Biomater.*, vol. 8, no. 1, pp. 274-281, 2012.

[6] X.C. Yin, F.Y. Li, Y.F. He, Y. Wanga, and R.-M. Wang, "Study on effective extraction of chicken feather keratins and their films for controlling drug release", *Biomater. Sci.*, vol. 1, no. 5, pp. 528-536, 2013.

[7] S. Hamasaki, A. Tachibana, D. Tada, K. Yamauchi, and T. Tanabe, "Fabrication of highly porous keratin sponges by freeze-drying in the presence of calcium alginate beads", *Mater. Sci. Eng. C*, vol. 28, no. 8, pp. 1250-1254, 2008.

[8] B. Srinivasan, R. Kumar, K. Shanmugam, U.T. Sivagnam, N.P. Reddy, and P.K. Sehgal, "Porous keratin scaffold–promising biomaterial for tissue engineering and drug delivery", *J. Biomed. Mater. Res. Part B: Appl. Biomater.*, vol. 92, no. 1, pp. 5-12, 2010.

[9] G. Cavallaro, M.R. Caruso, S. Milioto, R.F. Fakhrullin, and G. Lazzara, "Keratin/alginate hybrid hydrogels filled with halloysite clay nanotubes for protective treatment of human hair", *Int. J. Biol. Macromol.*, vol. 222, pp. 228-238, 2022.

[10] Y.E. Arslan, T.S. Arslan, B. Derkus, E. Emregul, and K.C. Emregul, "Fabrication of human hair keratin/jellyfish collagen/eggshell-derived hydroxyapatite osteoinductive biocomposite scaffolds for bone tissue engineering: from waste to regenerative medicine products", *Colloid. Surface. B: Biointerface.*, vol. 154, pp. 160–170, 2017.

[11] A. Tachibana, Y. Nishikawa, M. Nishino, S. Kaneko, T. Tanabe, and K. Yamauchi, "Modified Keratin Sponge: Binding of Bone Morphogenetic Protein-2 and Osteoblast Differentiation", *J. Biosci. Bioeng.*, vol. 102, no. 5, pp. 425-429, 2006.

[12] E. Ranjit, S. Hamlet, R. George, A. Sharma, and R.M. Love, "Biofunctional approaches of wool-based keratin for tissue engineering", *J. Sci.: Adv. Mater. Devices*, vol. 7, no. 1, pp. 100398, 2022.

[13] M. Zhai, Y. Xu, B. Zhou, and W. Jing, "Keratin-chitosan/n-ZnO nanocomposite hydrogel for antimicrobial treatment of burn wound healing: Characterization and biomedical application", *J. Photochem. Photobio. B: Biol.*, vol. 180, pp. 253-258, 2018.

[14] T.R. Ham, R.T. Lee, S. Han, S. Haque, Y. Vodovotz, J. Gu et al., "Tunable keratin hydrogels for controlled erosion and growth factor delivery", *Biomacromolecules*, vol. 17, pp. 225-236, 2016.

[15] N.M. Mahajan, K. Wanaskar, N. Ali, D.K. Mahapatra,

M. Iqbal, A.R. Bhat et al., "Innovative wound healing hydrogel containing chicken feather keratin and soy isoflavone genistein: in vivo studies", *Gels*, vol. 9, no. 6, pp. 462, 2023.

[16] L. Olariu, B.G. Dumitriu, C. Gaidau, M. Stanca, L.M. Tanase, M.D. Ene et al., "Bioactive low molecular weight keratin hydrolysates for improving skin wound healing", *Polymers*, vol. 14, no. 6, pp. 1125, 2022.

[17] J. Guo, S. Pan, X. Yin, Y.-F. He, T. Li, and R.-M. Wang, "pH-sensitive keratin-based polymer hydrogel and its controllable drug-release behavior", *J. Appl. Polym. Sci.*, vol. 132, no. 9, 2015.

[18] S.J. Min, J.S. Lee, H. Nah, H.-J. Moon, S.J. Lee, H.J. Kang et al., "Degradable and tunable keratin-fibrinogen hydrogel as controlled release system for skin tissue regeneration", *J. Bionic Eng.*, vol. 20, no. 3, pp. 1049-1059, 2023.

[19] B. Ledford, C. Barron, M.V. Dyke, and J.-Q. He, "Keratose hydrogel for tissue regeneration and drug delivery", *Semin. Cell Dev. Biol.*, vol. 128, pp. 145-153, 2022.

[20] I. Sinkiewicz, A. Śliwińska, H. Staroszczyk, and I. Kołodziejska, "Alternative methods of preparation of soluble keratin from chicken feathers", *Waste Biomass Valori.*, vol. 8, no. 4, pp. 1043-1048, 2017.

[21] R.-R. Yan, J.-S. Gong, C. Su, Y.-L. Liu, J.-Y. Qian, Z.-H. Xu et al., "Preparation and applications of keratin biomaterials from natural keratin wastes", *Appl. Micro. Biotechnol.*, vol. 106, no. 7, pp. 2349-2366, 2022.

[22] G.E. de Q. Souza, G.R.M. Burin, G.I.B. de Muniz, and H.J. Alves, "Valorization of feather waste in Brazil: structure, methods of extraction, and applications of feather keratin", *Environm. Sci. Pollut. Res.*, vol. 30, no. 14, pp. 39558-39567, 2023.

[23] F. Pourjavaheri, S. Ostovar Pour, O.A.H. Jones, P.M. Smooker, R. Brkljača, F. Sherkat et al., "Extraction of keratin from waste chicken feathers using sodium sulfide and L-cysteine", *Process Biochem.*, vol. 82, pp. 205-214, 2019.

[24] D.R. Goddard and L. Michaelis, "Studies on keratin", *J. Biol. Chem.*, vol. 106, pp. 605–614, 1934.

[25] T. Lingham-Soliar, *Microstructural Tissue-Engineering in the Rachis and Barbs of Bird Feathers*, Nature, 2017, pp. 1–13.

[26] L. Alibardi, L. Dalla Valle, V. Toffolo, and M. Toni, "Scale keratin in lizard epidermis reveals amino acid regions homologous with avian and mammalian epidermal proteins", *Anat. Rec. A Discov. Mol. Cell. Evol Biol.*, vol. 288, no. 7, pp. 734-752, 2006.

[27] R.D.B. Fraser and D.A.D. Parry, "Molecular packing in the feather keratin filament", *J. Struct. Biol.*, vol.

162, no. 1, pp. 1–13, 2008.

[28] Z. Meng, X. Zheng, K. Tang, J. Liu, and S. Qin, "Dissolution of natural polymers in ionic liquids: A review", *e-Polymers*, vol. 12, no. 1, 2012.

[29] N. Eslahi, F. Dadashian, and N. Hemmati, "Optimization of enzymatic hydrolysis of wool fibers for nanoparticles production using response surface methodology", *Adv. Powder Technol.*, vol. 24, nol. 1, pp. 416-426, 2013.

[30] P.S. Nigam, "Microbial enzymes with special characteristics for biotechnological applications", *Biomolecules*, vol. 3, no. 3, pp. 597-611, 2013.

[31] C. Tonin, M. Zoccola, A. Aluigi, A. Varesano, A. Montarsolo, C. Vineis et al., "Study on the conversion of wool keratin by steam explosion", *Biomacromolecules*, vol. 7, no. 12, pp. 3499-3504, 2006.

[32] J. Yin, S. Rastogi, A.E. Terry, and C. Popescu, "Self-organization of oligopeptides obtained on dissolution of feather keratins in superheated water", *Biomacromolecules*, vol. 8, no. 3, pp. 800-806, 2007.

[33] M. Zoccola, A. Aluigi, A. Patrucco, C. Vineis, F. Forlini, P. Locatelli et al., "Microwave-assisted chemical-free hydrolysis of wool keratin", *Text. Res. J.*, vol. 82, no. 19, pp. 2006-2018, 2012.

[34] X. Qin, C. Yang, Y. Guo, J. Liu, J.H. Bitter, E.L. Scott et al., "Effect of ultrasound on keratin valorization from chicken feather waste: Process optimization and keratin characterization", *Ultrason. Sonochem.*, vol. 93, pp. 106297, 2023.

[35] W.G. Crewther, R.D.B. Fraser, F.G. Lennox, and H. Lindley, "The chemistry of keratins", *Adv. Prot. Chem.*, vol. 20, pp. 191-346, 1965.

[36] Y.X. Wang and X.J. Cao, "Extracting keratin from chicken feathers by using a hydrophobic ionic liquid", *Process Biochem.*, vol. 47, no. 5, pp. 896-899, 2012.

[37] J. Zhu, N. Ma, S. Li, L. Zhang, X. Tong, Y. Shao et al., "Reinforced wool keratin fibers via dithiol chain re-bonding", *Adv. Funct. Mater.*, vol. 33, no. 14, pp. 2213644, 2023.

[38] H. Wieser, *The Use of Redox Agents in Breadmaking*, ed., 2nd Ed., Woodhead, 2012, pp. 447-469.

[39] K. Yamauchi and A. Yamauchi, "Preparation of stable aqueous solution of keratins, and physicochemical and biochemical properties of films", *Am. Chem. Soc. Polym. Prep. Div. Polym. Chem.*, vol. 39, no. 1, pp. 357-358, 1998.

[40] K. Katoh, T. Tanabe, and K. Yamauchi, "Novel approach to fabricate keratin sponge scaffolds with controlled pore size and porosity", *Biomaterials*, vol. 25, no. 18, pp. 4255–4262, 2004.

[41] A. Nakamura, M. Arimoto, K. Takeuchi, and T. Fujii, "A rapid extraction procedure of human hair proteins and identification of phosphorylated species", *Biol. Pharm. Bull.*, vol. 25, no.5, pp. 569-572, 2002.

[42] P.M. Schrooyen, P.J. Dijkstra, R.C. Oberthu, A. Bantjes, and J. Feijen, "Partially carboxymethylated feather keratins. 1. Properties in aqueous systems", *J. Agr. Food Chem.*, vol. 48, no. 9, pp. 4326-4334, 2000.

[43] P.M.M. Schrooyen, P.J. Dijkstra, R.C. Oberthür, A. Bantjes, and J. Feijen, "Stabilization of solutions of feather keratins by sodium dodecyl sulfate", *J. Colloid Interf. Sci.*, vol. 240, no. 1, pp. 30-39, 2001.

[44] A.J. Poole, R.E. Lyons, and J.S. Church, "Dissolving feather keratin using sodium sulfide for bio-polymer applications", *J. Polym. Environ.*, vol. 19, pp. 995-1004, 2011.

[45] Y. Esparza, A. Ullah, Y. Boluk, and J. Wu, "Preparation and characterization of thermally crosslinked poly(vinyl alcohol)/feather keratin nanofiber scaffolds", *Mater. Des.*, vol. 133, pp. 1-9, 2017.

[46] U.K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4", *Nature*, vol. 227, no. 5259, pp. 680-685, 1970.

[47] F. Happey and R.L. Wormell, "53-Regenerated keratin fibres from wool", *J. Text. Inst. Transact.*, vol. 40, no. 12, pp. T855-T869, 1949.

[48] P. Sen, C. Arun, and J. Divvyapriya, "A pilot-scale study on the extraction and optimization of keratin from human hair–an adapted strategy for the control of environmental menace", *J. Environ. Treat. Tech.*, vol. 9, no. 1, pp. 342-348, 2021.

[49] N.B. Kamarudin, S. Sharma, A. Gupta, G.K. Chua, S.M.S. Bin Tuan Chik, and R. Gupta, "Statistical investigation of extraction parameters of keratin from chicken feather using design-expert", *3 Biotech*, vol. 7, no. 2, pp. 127, 2017.

[50] K.T. Welu, S.M. Beyan, S. Balakrishnan, and H. Admassu, "Chicken feathers based Keratin extraction process data analysis using response surface-box-Behnken design method and characterization of keratin product", *Current Appl. Sci. Technol.*, pp. 163-177, 2020.

[51] T. Yan, T.X. Li, and R.Z. Wang, "Thermochemical heat storage for solar heating and cooling systems", *Adv. Sol. Heat. Cool.*, pp. 491-522, 2016.

[52] C. Breese Jones and D.K. Mecham, "The dispersion of keratins. 1. Studies on the dispersion and degradation of certain keratins by sodium sulphide", *Arch Biochem.*, vol. 2, pp. 209-223, 1943.

[53] A. Aluigi, C. Vineis, A. Varesano, G. Mazzuchetti, F. Ferrero, and C. Tonin, "Structure and properties of

keratin/PEO blend nanofibres", *Eur. Polym. J.*, vol. 44, no. 8, pp. 2465-2475, 2008.

[54] S. Isarankura Na Ayutthaya, S. Tanpichai, and J. Wootthikanokkhan, "Keratin extracted from chicken feather waste: extraction, preparation, and structural characterization of the keratin and keratin/biopolymer films and electrospuns", *J. Polym. Environ.*, vol. 23, no. 4, pp. 506-516, 2015.

[55] K. Wang, R. Li, J.H. Ma, Y.K. Jian, and J.N. Che, "Extracting keratin from wool by using L-cysteine", *Green Chem.*, vol. 18, no. 2, pp. 476-481, 2016.

[56] D.J. Lyman, J. Murray-Wijelath, and M. Feughelman, "Effect of temperature on the conformation of extended α-keratin", *Appl. Spectrosc.*, vol. 55, no. 5, pp. 552-554, 2001.

[57] A. Idris, R. Vijayaraghavan, U.A. Rana, D. Fredericks, A.F. Patti, and D.R. MacFarlane, "Dissolution of feather keratin in ionic liquids", *Green Chem.*, vol. 15, no. 2, pp. 525-534, 2013.

[58] A. Ghosh, S. Clerens, S. Deb-Choudhury, and J.M. Dyer, "Thermal effects of ionic liquid dissolution on the structures and properties of regenerated wool keratin", *Polym. Degrad. Stab.*, vol. 108, pp. 108-115, 2014.

[59] M. Zoccola, A. Aluigi, and C. Tonin, "Characterisation of keratin biomass from butchery and wool industry wastes", *J. Mol. Struct.*, vol. 938, no. 1-3, pp. 35-40, 2009.

[60] N.A. Azmi, A. Idris, and N. Saadah, "Ultrasonic Technology for Value Added Products from Feather Keratin", *Ultrason Sonochem.*, 2018. doi: 10.1016/j.ultsonch.2018.04.016

[61] Y.S. Lee, L.Y. Phang, S.A. Ahmad, and P.T. Ooi, "Microwave-alkali treatment of chicken feathers for protein hydrolysate production", *Waste Biomass Valoriz.*, vol. 7, no. 5, pp. 1147-1157, 2016.

[62] I.A. Aguayo-Villarreal, A. Bonilla-Petriciolet, V. Hernández-Montoya, M.A. Montes-Morán, and H. Reynel, "Batch and column studies of Zn^{2+} removal from aqueous solution using chicken feathers as sorbents", *Chem. Eng. J.*, vol. 167, nol. 1, pp. 67-76, 2011.

[63] Y. Zhang, W. Zhao, and R. Yang, "Steam flash explosion assisted dissolution of keratin from feathers", *ACS Sustain. Chem. Eng.*, vol. 3, no. 9, pp. 2036-42, 2015.

[64] M.J. Buehler and S. Keten, "Elasticity, strength and resilience: A comparative study on mechanical signatures of α-helix, β-sheet and tropocollagen domains", *Nano Res.*, vol. 1, pp. 63-71, 2008.

[65] E.J.C. Amieva, C. Velasco-Santos, A.L. Martínez-Hernández, J.L. Rivera-Armenta, A.M. Mendoza-Martínez, V.M. Castaño et al., "Composites from chicken feathers quill and recycled polypropylene", *J. Compos. Mater.*, vol. 49, no. 3, pp. 275-283, 2015.

[66] M.A. Khosa and A. Ullah, "In-situ modification, regeneration, and application of keratin biopolymer for arsenic removal", *J. Hazard. Mater.*, vol. 278, pp. 360-371, 2014.

[67] R. Li and D. Wang, "Preparation of regenerated wool keratin films from wool keratin-ionic liquid solutions", *J. Appl. Polym. Sic.*, vol. 127, no. 4, pp. 2648-2653, 2013.