Research Article

Optimizing the Extraction of Keratin from Cattle Hoof Using Central Composite Design

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Cattle hoofs are abundantly available by-product sources of organic material from the slaughterhouse. It can be successfully converted into keratin protein. Keratin extracted using alkali hydrolysis has a better conservancy of keratin structure. The purpose of this study is optimizing the extraction of keratin from cuttle hoof. Designing of the experiment, analysis of the results, and optimization of the process parameters have been conducted by central composite design (CCD). Three factors (temperature (A), time (B), and concentration of NaOH (C)) each at five levels have been used to extract the keratin protein from cattle hoof and two response variables (dissolution % and purity %) have been considered in the study. The results obtained demonstrate that extraction temperature, alkali concentration, and time showed a significant effect on the purity and dissolution of keratin. The regression model shows that all factors have positive and significant relation with dissolution percentage whereas only temperature and concentration of NaOH have significant and negative relation with purity percentage. It is observed that the Biuret and Fourier-transform infrared spectroscopy (FTIR) tests showed better preservation of protein structure in extractive keratin. The FTIR spectra, indicates that amide I and amide II occur at a wave length of 1633 and 1542 cm^{-1} for raw cattle hoof and at wave length of 1650 and 1542 cm^{-1} for keratin protein, respectively. Thus, it can be concluded that the cattle hoof by-product could offer an alternative keratin source. Finally, the extraction process had an optimum value of 0.5 M NaOH, 60 minutes reaction time, and 55°C temperature with 85% dissolution and 89.6% purity.

1. Introduction

Activities in slaughterhouses generate a high level of organic waste, such as cattle hoof, horn, hide, and bone that directly or indirectly affects the health of residents living in the vicinity. Moreover, hooves are a hard keratin source that has very slow decomposition and pollutes the environment for a long period by releasing foul smells. In fact, the presence of a strong disulphide covalent bond in keratin molecule of the keratinous material makes it to be resistant to a common enzyme and chemical attack (long-time degradation) and becomes insoluble in water, weak acid, alkali, and most organic solvent [1, 2].

In valorisation of those keratinous wastes by keratin polymer extraction, some of common ways that has been used include, chemicals (reduction and oxidation) [3–6] enzyme and microbial [7, 8], and microwave methods [4, 9]. New green solvent called ionic liquid is also another good means of a human hair [10], wool, camel hair, and chicken feather [11] keratin dissolution, while it has low extraction

rate [12]. The solubilization is by reducing the stability of keratin by straightening out of disulphide bonds and hydrogen bonds to access agent to dissolve the protein into solution [13]. There has been growing interest in the use of naturally derived keratin polymer for a multitude of purposes, which is applicable in food, cosmetics, medical, composite, textile, and agriculture industries [6, 14–16].

The chemical composition of a cattle hoof shows that it contains largely α-helical conformation with a mixture of β-sheet [5], around 90% crude protein [17], therefore, it is valuable to extract keratin from hoof for biomedical, cosmetics, and health care applications [18]. About 1.5 billion of cattle are slaughtered every year from meat and from each cattle four pieces of hooves are obtained [19] As a result of this, extraction of keratin from cattle hooves has sustainable supply of raw material. Some research demonstrates that cattle hoof yields the highest keratin protein than wool, feather, and hair keratin [16, 20]. Potential keratin extraction from animal hooves tissues was introduced in 1860 [21, 22]. Up to date, keratin from cattle hoofs is extracted using a reducing agent [16, 20, 23] and enzymatic method [4]. Hence, alkaline hydrolysis is a good alternative for the extraction of keratin because of its effectiveness and simplicity with a lower loss of amino acids [8]. During the extraction process, sodium hydroxide (NaOH) is used as the main solubilizing agent and urea as denaturing agent [22].

In the alkali hydrolysis process, many factors could affect the extraction process and properties of keratin extractive, however, in this article extraction time, temperature, and concentration of alkali were taken as important factors affecting the extraction process of keratin from biomass [24, 25]. Researchers indicate that having heating aided hydrolysis provides a better yield while, higher heating temperature leads to the destruction of amino acids [2]. To the best of our knowledge, this extraction method has not been used for the digestion of cattle hoof and even the effects of extraction variables on the keratin yield and purity of the keratin have not been well studied. Thus, the current work aims to extract keratin from cattle hoof using sodium hydroxide solvent and urea through a thermo-chemical process, and then optimize the process to get an experimental condition with optimum yield/dissolution and purity of keratin.

2. Materials and Methods

2.1. Materials.
Cattle hoofs were collected from a slaughterhouse in the Bahir Dar city, Ethiopia. Laboratory grade sodium hydroxide 99.8% (NaOH), hydrochloric acid 36%·w/w (HCl), and Urea 99.0% (NH_2CONH_2) were obtained from the Ethiopian Institute of Textile and Fashion Technology, chemical. The main instrument used in this study was a Freeze drier (BK-F01/05), hammer crusher, and Fourier-transform infrared spectroscopy (JASCO FR/IR 6600).

2.2. Methods

2.2.1. Proximate Analysis of Cattle Hoof.
After collection, the hoof was washed with distilled water to remove impurities and dried at 100°C for 5 hours in an oven

before further process. As shown in Figure 1, the dried cattle hoof was crushed to 10–20 mm diameter by a hammer crusher and pulverized into 1 mm mesh size and below as indexed by [26–28]. The pulverized hoof was then collected in zipper plastic bags and stored at the standard atmospheric condition for further proximate analysis.

2.2.2. Determination of Fat Content.
A minimum standard weight of two grams of Pulverized cattle [26] was used. It is then further soaked with diethyl ether and allowed to stand for 2 hours without stirring for sedimentation [16, 27, 29] and dried at 50°C for 24 h. The fat content of the raw hoof is determined by the following equation:

$$Fat\ content\ (\%) = \frac{Weight\ of\ fat * 100}{Weight\ of\ sample}. \tag{1}$$

2.2.3. Determination of Moisture Content.
A hot air oven was used to determine the moisture content of the samples. Two grams of sample were processed according to ASTM D1576-90 standard, and then average moisture content was determined by the following equation:

$$Moisture\ content\ (\%) = \frac{(W1 - W2) * 100}{W1}, \tag{2}$$

Where, W1 is the weight (g) of the sample before drying and W2 is the weight (g) of the sample after drying.

2.2.4. Determination of Ash Content.
Ash is the inorganic residue obtained after the combustion of hoof biomass. The ash content of the raw hoof was calculated in relation to the dry weight of the original sample after 4 hr ignition of the sample at 575°C using [30]. The ash content of the raw hoof was determined by the following equation:

$$Ash\ content\ (\%) = \frac{Weight\ of\ ash * 100}{Weight\ of\ sample}. \tag{3}$$

2.2.5. Determination of Protein Content.
The protein content was determined by the Kjeldahl method according to [30] using the official method 979.09. This method has been used in nitrogen determination, and then multiplied by a conversion factor as determined by the following equation:

$$Protien\ content\ (\%) = Nitrogen\ content\ in\ sample * 6.25. \tag{4}$$

2.2.6. Alkali Extraction of Keratin.
The defatted hoof keratin was extracted and solubilized using 0.3–1 M of NaOH and 0.64 g of urea. During the extraction of keratin, where the agents solubilize the keratin, alkali media was importantly used [31]. Once the keratin was solubilized, turbid in a solution then filtered using a polyester cloth [28]. The solution from the filtrate was precipitated at acidic media (pH
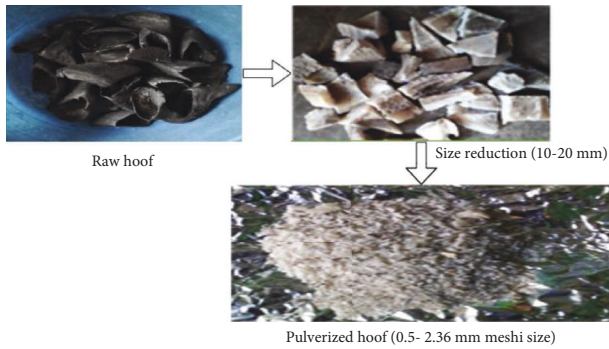
Figure 1: Cattle hoof pulverization procedure.

Table 1: Actual levels of design factors (CCD).

Factors	Units	Low	Center point	High
Temperature	°C	55	65	75
Time	Min	60	120	180
Concentration	M	0.3	0.65	1

4) using isoelectric precipitation of the protein of 1 N HCl [32, 33], and then freeze-dried to obtain recovered keratin powder [34].

To find out the dissolution percentage of the extraction process, the weight of undissolved residue has been identified, once it is oven-dried until constant weight has been obtained [35]. While the purity of the keratin powder was identified using the Kjeldahl method [30]. The actual Design factors levels using CCD are illustrated in Table 1.

2.2.7. Characterization of Extracted Keratin

(1) Biuret Test. One molarity copper sulphate solution and sodium hydroxide solution were prepared separately. The solution collected was mixed with sodium hydroxide solution by 1 : 1 ratio. Three drops of copper sulphate solution were added to the mixture solution. The color changes in the solution were observed and recorded [27].

(2) FTIR Spectroscopy. The functional groups present in extracted keratin powder could be recorded using FT-IR (JASCO FT/IR 6600). About 2 mg of the sample would be taken and prepared using KBr, which is characterized under the spectra range 4000–400 cm^{-1} in a resolution of 4 cm^{-1}, 400 scans. Infrared radiation has been made to pass through the sample and the resulting spectrum was representing the molecular transmission, creating a molecular fingerprint of a sample.

2.2.8. Analysis and Optimization.

Central composite design (CCD) has been used to design the experiment, analyze the results of the experiment and optimization of the process. Central composite design is an experimental design, useful in response surface methodology. In this design, the center points are augmented with a group of axial points called star points. With this design, quickly first-order and second-order terms can be estimated. Five levels have been taken for all three factors (low, center point, high, $+\alpha$, and $-\alpha$). For each experiment, the average of five replicate have been taken. The optimization of operational factors such as temperature (°C), time (min), and concentration of NaOH (M) is necessary in order to decrease the number of experimental tests and determine the optimum conditions for input factors. In this regard, the response surface methodology (RSM) coupled with central composite design (CCD) (CCD-RSM design) is an efficient tool that has been used to model the correlation between output response and input of the machining process [19].

3. Result and Discussion

3.1. Proximate Analysis

3.1.1. Parameter. The pulverized raw hoof had an average of fat, protein, ash, and moisture content as shown in Table 2. Based on the result about 1.5% crude fat content was determined by soaking the milled hoof sample with diethyl ether. The crude fat content of the raw sample was 1.47%, which is in agreement with Falaye and Sule, (2020).

The average value of ash was 6.55%, which is higher than the report made for cattle hoof meal [17]. Higher ash content is usually an implication of higher impurity or inorganic residue. About 75.0534% protein content was achieved through Kjeldahl analysis, which is relatively lower than the report made by [17].

The average moisture content of the hoof is 5.96%. Hence, it was a bit lower value than the report by [3] and a bit proximate to the report made by [36]. The capability of cattle hoof to absorb moisture from the atmosphere has a vital inference for the storage, transport, processing, and durability of materials, which cause damage due to microorganism contamination [36]. As the moisture content in the raw hoof does not exceed 10.5%, it is a good implication of a material stored for a long time with a minimum microbial growth [28].

3.2. Alkali Extraction and Optimization

3.2.1. Alkali Extraction. Hoof keratin was extracted and solubilized using different concentrations of caustic soda at different extraction times and temperatures. The solubilized keratin was precipitated and freeze-dried as shown in Figures 2(a) and 2(b), respectively. Freshly solubilized keratins are prone to precipitation because of their hydrophobicity and disulphide bridges tendency to reassemble from the newly resulted sulfhydryl groups. As a consequence, a complex mixture of supramolecular aggregates results, which tend to associate and separate as flocks [37].

Hoof alkali hydrolysis experiment performed on various process parameters has a variability of output (Table 3) and the order of the experiments are taken in random. The dissolution and purity percentages were 79–99.34% and 69.1–95.25%, respectively. The lowest (79%) and highest (99.341%) dissolution percentages were achieved at the extraction condition of 55°C, 60 min, 0.3 M, and 180 min, 75°C, and 1 M, respectively. Dissolution percentage of keratin mainly because of the solubility and cleavage of peptide and disulphide bond. The solubility and extent of

TABLE 2: Proximate analysis of cattle hoof.

Parameter	Mean	Standard deviation
Protein content (%)	75.05	0.01
Fat content (%)	1.5	0.002
Ash content (%)	6.554	0.18
Moisture content (%)	6.15	1.46

(a)   (b)

FIGURE 2: (a) Precipitated protein and (b) powder keratin after freeze-drying and grinding.

TABLE 3: Dissolution and purity of extractive keratin at different conditions.

Run	Factor 1 A: Temperature(°C)	Factor 2 B: Time (min)	Factor 3 C: Concentration of NaOH (M)	Response 1 dissolution (%)	Response 2 purity (%)
1	75	60	0.3	93.69	82
2	55	60	0.3	78	95.2
3	75	180	0.3	94.77	78.08
4	65	120	0.65	94.31	80.18
5	65	120	0.65	96.5	81.29
6	75	180	1	99.34	82.45
7	65	19.09	0.65	92.56	81.51
8	75	60	1	97.53	73.83
9	55	180	1	96.09	79.78
10	65	120	0.65	94.78	79.89
11	55	60	1	91.96	75.98
12	65	120	0.65	95	78.56
13	65	120	0.65	93.57	80
14	65	220.91	0.65	96.68	81.5
15	65	120	0.65	95.56	80.21
16	65	120	1.24	96.62	69.10
17	55	180	0.3	80.22	84.57
18	81.82	120	0.65	99.19	80
19	48.18	120	0.65	85.9	88.19

dissolution are mainly a function of NaOH concentration and temperature [32].

In the meantime, the lowest (69.1%) and higher (95.25%) purity percentage of the extractives was found at the extractive condition of 120 min and 65°C, 1.24 M and 60 min, and 55°C and 0.3 M, respectively, from the experimental trials as shown in Table 3. Even if, the experiment results yield higher at higher temperature and concentration it is not true for a purity, because of the keratin polymer structure is disturbed at these experimental conditions. The experimental trial also shows that having a minimum extraction temperature of 48.18°C is not guaranty for higher purity, in which it lowers the conversion of cattle hoof in to

keratin polymer. The purity of the extractives was highly affected by the concentration and temperature of the hydrolysis process. This is in fact due to the environment of the process affecting the degradation of biopolymer [11]. As the temperature and NaOH concentration increase vigorously there is a loss of keratin structure and even all protein being in the amino acid level.

3.3. Analysis of the Significance of Factors on Dissolution Percentage. Based on the ANOVA in Table 4, the model generated for hoof keratin extraction was significant (P-value is less than 0.05). This is also true for a model term of

TABLE 4: Analysis of variance (ANOVA) for dissolution and purity percentage of keratin.

	Source	Sum of squares	Df	Mean square	F-value		P-value
	Model	597.03	9	66.34	71.35	<0.0001	Significant
	A	276.15	1	276.15	297.00	<0.0001	
	B	19.16	1	19.16	20.61	0.0014	
	C	208.66	1	208.66	224.41	<0.0001	
Dissolution percentage	AC	57.27	1	57.27	61.60	<0.0001	
	A^2	10.98	1	10.98	11.81	0.0074	
	C^2	47.21	1	47.21	50.77	<0.0001	
	Residual	8.37	9	0.9298			
	Lack of fit	3.25	4	0.8118	0.7926	0.5772	Not significant
	Source	Sum of squares	df	Mean square	F-value		P-value
	Model	498.95	9	55.44	58.46	<0.0001	Significant
	A-temperature	79.75	1	79.75	84.10	<0.0001	
	C-concentration NaOH	123.22	1	123.22	129.94	<0.0001	
	AB	16.75	1	16.75	17.66	0.0023	
Purity percentage	AC	51.24	1	51.24	54.03	<0.0001	
	BC	91.22	1	91.22	96.20	<0.0001	
	A^2	37.12	1	37.12	39.14	0.0001	
	B^2	7.26	1	7.26	7.66	0.0218	
	C^2	13.25	1	13.25	13.98	0.0046	
	Residual	8.53	9	0.9483			
	Lack of fit	4.71	4	1.18	1.54	0.3193	Not significant

TABLE 5: Fit statistics.

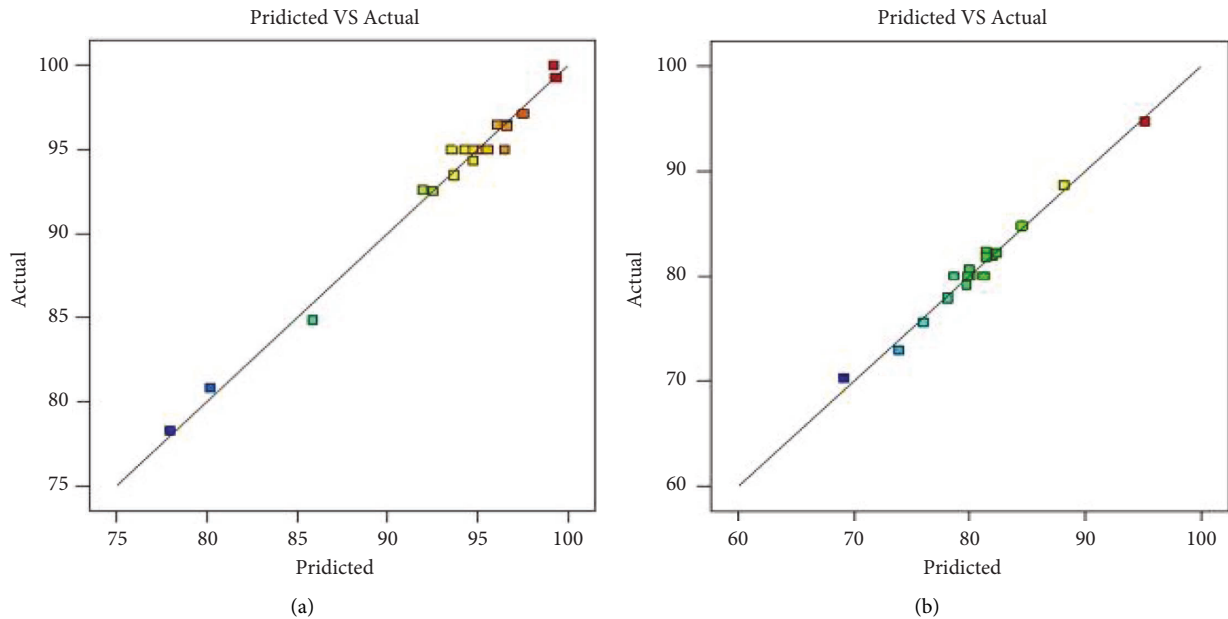|				
Fit statistics for dissolution percentage	C.V. %	1.03	Adjusted R^2	0.97
	R^2	0.99	Predicted E^2	0.93
Fit statistics for purity percentage	C.V. %	1.21	Adjusted R^2	0.97
	R^2	0.98	Predicted R^2	0.90

(a)

(b)

FIGURE 3: Predicted Vs actual experimental value for (a) dissolution and (b) purity percentage.

temperature, time, concentration, a combination of temperature and concentration of NaOH, temprature^2, and concentration^2, but not for a combined effect of time with temperature and concentration and time^2. Lack of fit F-value (0.79) is not significant and its insignificance was importantly been good.

As indicated by Table 5, the predicted R square (0.9328) and adjusted R square (0.9724) for dissolution percentage of keratin were in agreement with each other (their difference is less than 0.2). The quality of the fit model for the developed correlations was stated by R squared (0.9862). It implies that

experimental variables considered, can attribute about 98.62% to the extraction process. This shows that the regression model equations successfully captured the correlation between process parameters to the dissolution of product, in which all the points are close to the line of perfect fit as shown in Figure 3(a).

The lower coefficient of variation (CV = 1.03%) indicates that the results of the fitted model are reliable. The value of the adjusted coefficient (0.9724) is also very high, supporting the significance of the model. As the fitted model provides a good approximation to the experimental condition, the model was employed to find the values of the process variables for optimum dissolution percentage of hoof keratin.

Model (5) shows the empirical relation between dissolution percentage of hoof keratin with factors considered in the extraction process (A = Temperature, B = time, and C = concentration of NaOH, and AC = interaction of temperature concentration of NaOH). As equation (5) clearly shows, dissolution percentage increases as all the factors increase and it increases as the interaction effect AC, and the quadratic terms, A^2, and C^2 decrease. There is a positive relation between dissolution percentage of keratin and temperature, time, and concentration of NaOH, where as it has negative relation with interaction (temperature and concentration of NaOH), quadratic effect of temperature and concentration of NaOH.

$$
\begin{aligned}
Dissolution = &-28.9345 + 2.19639A + 0.0671607B \\
&+ 86.7593C - 0.764464 * AC+ \\
&- 0.00894952 A^2 - 19.3421C^2.
\end{aligned}
\tag{5}
$$

3.4. Analysis of the Significance of Factors on Purity Percentage. As shown in Table 4 the model is significant for analysis of keratin purity percentage, as model F- value is 58.46. All the factors, the interaction of the factors, and quadratic terms are significant ($P < 0.05$) except time at a 95% significance level. As the model is expected to fit, the nonsignificance of lack of fit F-value was importantly good as shown in Table 4.

As indicated by Table 5 the predicted R square (0.8955) and adjusted R square (0.9664) for purity percentage of keratin agreed with each other as their difference is less than 0.2. From R squared (0.9832) value, the contribution of experimental variables for the extraction process is about 98.32%, in which all the points are close to the line of a perfect fit. So, the developed model equation successfully captures the correlation between process parameters and the purity of the product.

The relation between experimental and predicted values was good as presented in Figure 3(b). As the coefficient of variation (1.21%) is lower, it is possible to confirm the reliability of the fitted model. A higher value of the adjusted coefficient (0.9664), supports the significance of the model. As the fitted model provides a good approximation to the experimental condition, the model was employed to find the values of the process variables for optimum purity percentage of keratin protein.

Model (6) shows the empirical relation between the purity percentage of hoof keratin with factors considered in the extraction process (A, B, and C). As clearly indicated in the equation, all the factors and C^2 have a negative relation with the response (purity) and all interaction effects, and quadratic effects such as A^2, and B^2 have a positive relationship with the response.

$$
\begin{aligned}
Purity = &+232.91018 - 3.13999A - 63.5783C \\
&+ 0.00241167 * AB + 0.723071 * AC \\
&+ 0.160798 * BC + 0.016453 A^2 \\
&+ 0.000202 B^2 - 10.24893C^2.
\end{aligned}
\tag{6}
$$

The effect of temperature on the dissolution percentage of hoof keratin was presented in Figure 4(a), with a temperature range from 55 up to 75°C. As shown in Figure 4(a), the dissolution possesses a constructive relationship with that of temperature, and as the temperature increased the dissolution percentage was increased, which is in agreement with [38]. As mentioned in Figure 4(b) purity of keratin extractives under those temperature ranges was plotted. As reaction temperature is increased, the purity of the extractives was reduced.

In fact, heating aided hydrolysis provides a better dissolution while, higher heating temperature leads to the destruction of amino acids [2]. Also, as the temperature increase from 55 to 75°C, the reaction process can get much energy to accelerate the reduction of the chemical and physical state of the hoof. While lowering the extraction temperature causes a lower reduction of the disulphide bond, not the peptide bond.

Figure 5(a) presents the effect of varying the extraction time from 60–180 min on the dissolution percentage of hoof keratin. As illustrated in Figure 5(a), increasing the extraction time increases the dissolution percentage. Generally speaking, increasing extraction time shows an improvement in dissolution percentage.

The purity of the extractive keratin is reduced as extraction time increased from 60 min to 80 min while the effect is not substantial as shown in Figure 5(b). At lower extraction time the purity was comparatively higher than at the larger time of extraction. Long-time extraction causes the loss of keratin structure (amino acid) and reduces purity.

At lower concentrations, the reaction process had lower keratin dissolution and vise versal (Figure 6(a)). This is because at lower concentrations there is a high solid to liquid ratio and aggregation of the hoof material, which reduces the dissolution [38]. There is a direct relationship between the concentration of alkali with the extent of keratin solubility [10, 39].

The purity of the extractive reduced significantly as the concentration of NaOH changes from 0.3–1 M as shown in Figure 6(b). Higher dissolution at higher NaOH concentration may cause some extra dissolved material from hoof possessed in a liquid state. In another way, the increasing concentration also causes degradation of the keratin protein, which affects the purity undesirably.
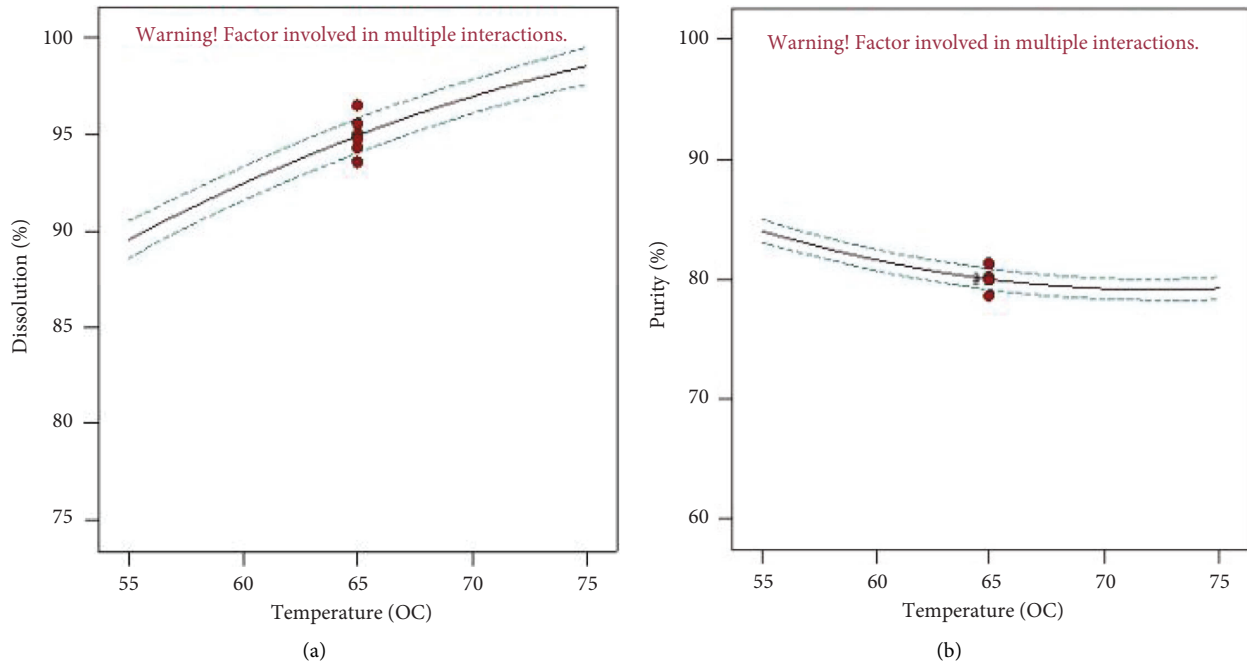
(a)

(b)

FIGURE 4: Effect of temperature on (a) dissolution and (b) purity of keratin.
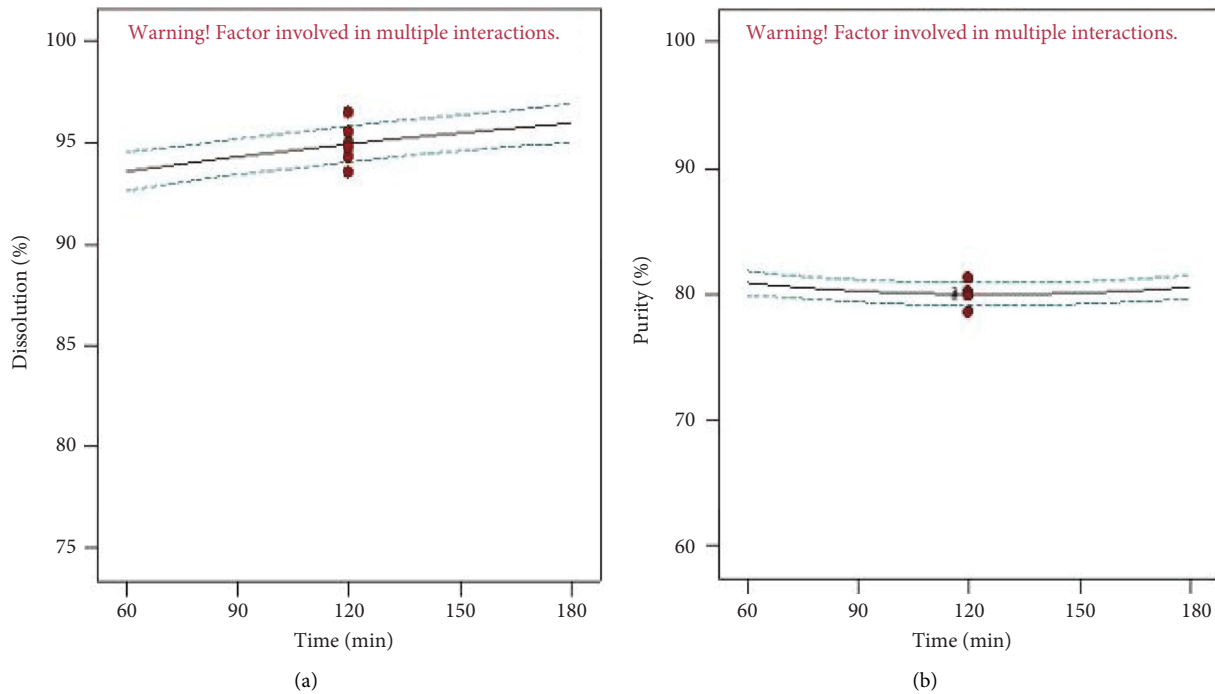

(a)

(b)

FIGURE 5: Effect of extraction time on (a) dissolution and (b) purity of hoof keratin.

3.5. Response Surface Analysis.

The three-dimensional response surface plot as a function of the interaction of two factors on dissolution and purity percentage is indicated in Figures 7 and 8, respectively.

The interaction effect of the factors as shown in Figures 7(a)–7(c), affect the dissolution positively, as both of those factors were increased the dissolution percentage increased. This is because, a fast extraction process and solubility were achieved at higher temperatures, concentrations, and time [38].

The purity of the polymer decreased as temperature and time increased, even if the decrement in purity by time factor is insignificant as shown in Figures 4(b) and 5(b). While the interaction effect of each factor is different from the separate effect of factors as can be seen in Figure 8(a). Figures 4(b) and 6(b) show that, the reaction temperatures and
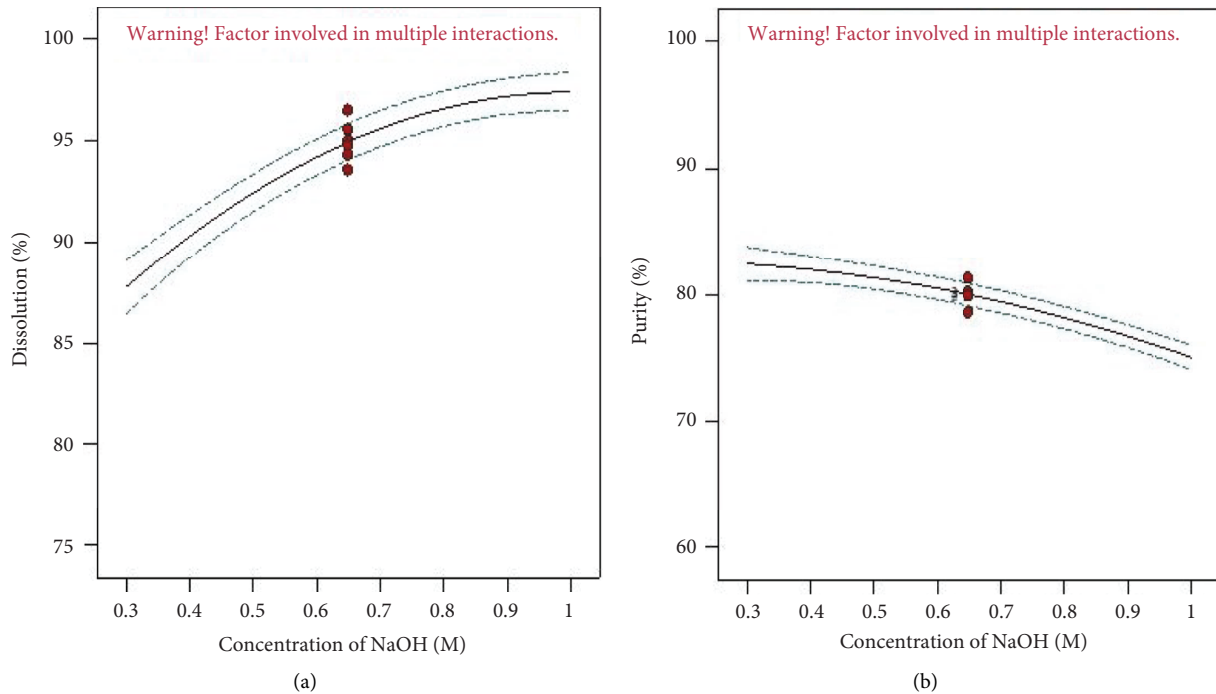
(a)

(b)

FIGURE 6: Effect of NaOH concentration on (a) dissolution percentage and (b) purity of keratin.

concentration harm keratin purity. While the interaction effect of those factors shows the probability of getting little improvement in purity (Figure 8(b)).

3.6. Numerical Optimizations. The optimum condition for the extraction of keratin protein from cattle hoof was found to be 0.5 M of NaOH concentration, time of 60 minutes and 55°C temperature with optimum desirability of 85% for dissolution and 89.586% for purity.

3.7. Characterization of Keratin

3.7.1. Biuret Test. The presence of protein in keratin extractive solution was confirmed by the biuret test [27]. After the reagent was added, the color of the extractive solution is changed to purple. This is true only if peptide bonds are present in the solution [40]. The higher the peptide bond in the solution, the higher intensity of purple color possessed.

As shown in Figure 9, a darker purple color was offered in sample "a" using 0.65 M, 65°C, and 120 min of the extraction process. While sample "b" and sample "c" are extracted at a minimum concentration (0.061373 M) and maximum concentration (1 M), respectively, shift from purple to blue-purple and pink-purple color, respectively. Therefore, having a higher or lower value of NaOH concentration greatly affects the experimental process or the amount of protein being extracted in the solution. This analysis has also been in agreement with ANOVA analysis of factors on purity and dissolution percent.

3.7.2. Fourier Transform Infrared (FTIR). Numerous characteristic bands have been possessed by keratin, such as amide A (N-H stretching), amide I, amide II, and amide III
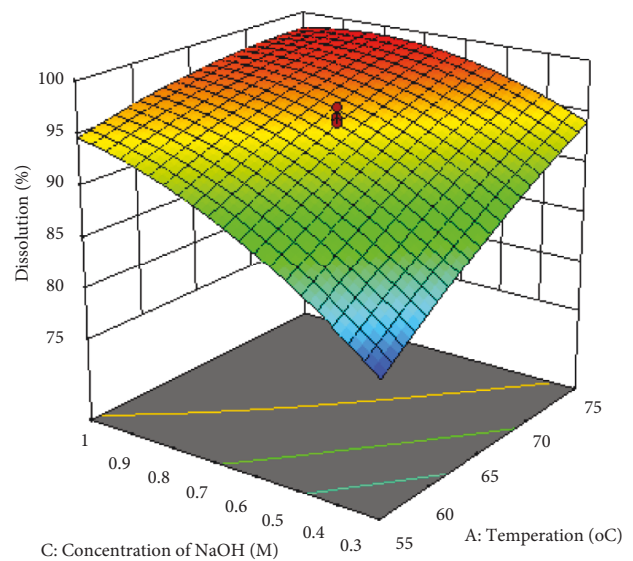

FIGURE 7: 3-D plot for dissolution percentage showing the interaction effect of temperature and concentration.

[41]. Important information about protein conformation and change in protein backbone structure is given by Amide I–III bands. Amide I is mainly originating from the stretching vibration of C=O at the characteristic band from 1700 to 1600 cm^{-1} [1]. It is widely related to the analysis of the secondary structure of a protein [42].

While amide II is observed at the characteristic band from 1560 to 1500 cm^{-1}, which is mainly from N-H bending and C-N stretching vibration. The presence of both α-helix and β-sheets structure in hoof keratin resembles bird and reptile structural conformation [43]. Amide III is also
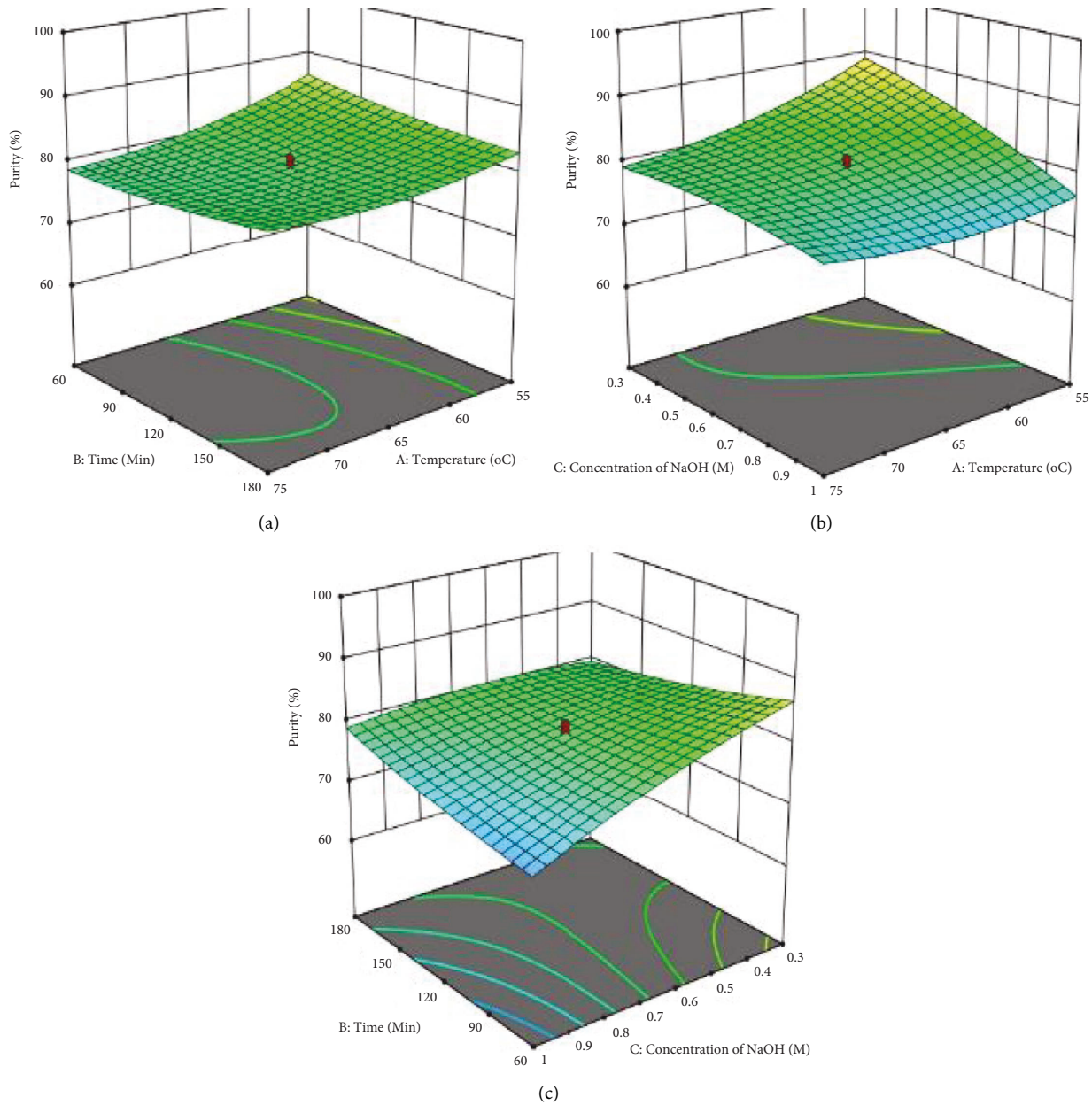
(a)

(b)

(c)

FIGURE 8: 3-D plot for purity percentage showing the interaction effect of (a) temperature and time, (b) temperature and concentration, and (c) time and concentration.

present around 1276 and the presence of this band is due to the presence of beta structure [44]. The FTIR spectra shown in Figure 10, indicate amide I and amide II at 1633 and $1542 \, cm^{-1}$ for raw cattle hoof and 1650 and $1542 \, cm^{-1}$ for keratin protein, respectively.

The characteristic band of both α-helix and β-sheets are observed in the amide I as well as in amide II. An α-helix conformation in the amide I and amide II are from $1657–1650 \, cm^{-1}$ and $1550–1540 \, cm^{-1}$, respectively [28]. While the β-sheets structure of keratin was observed from $1635–1615 \, cm^{-1}$ and $1535–1520 \, cm^{-1}$ in the amide I and amide II region. In raw cattle hoof, α-helix and β-sheets conformation been appeared at 1658, 1650, 1633, and $1623 \, cm^{-1}$ in the amide I region and 1545 and

$1524 \, cm^{-1}$ in the amide II region as shown in Figure 11. While for keratin powder, the expanded region shows a broader band for α-helix conformation at 1650 and $1532 \, cm^{-1}$ in the amide I and amide II region, respectively. As shown in Figure 10 presence of amide III around $1276 \, cm^{-1}$ confirms the presence of beta configuration in keratin.

Despite amide I, amide II, and amide III, N-H stretching vibration of the peptide bond (-CO-NH-) can be observed at a wavelength range of $3310–3270 \, cm^{-1}$ [20, 41]. In the present scenario, N-H stretching occurs at $3291 \, cm^{-1}$ for both raw hoof and powdered keratin (Figure 11). This shows the nondenaturing of the folded keratin structure at the time of the experimental process.
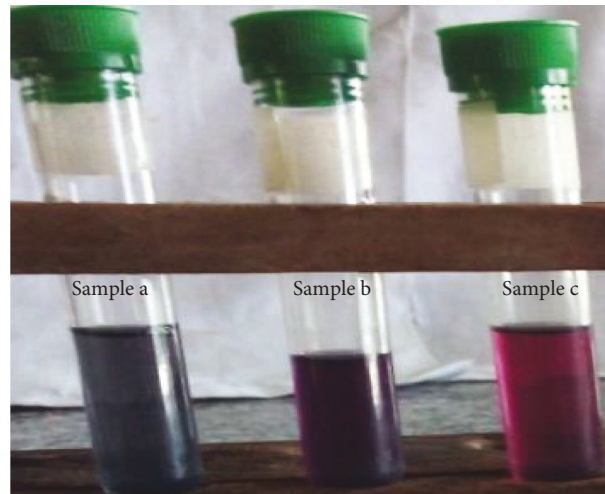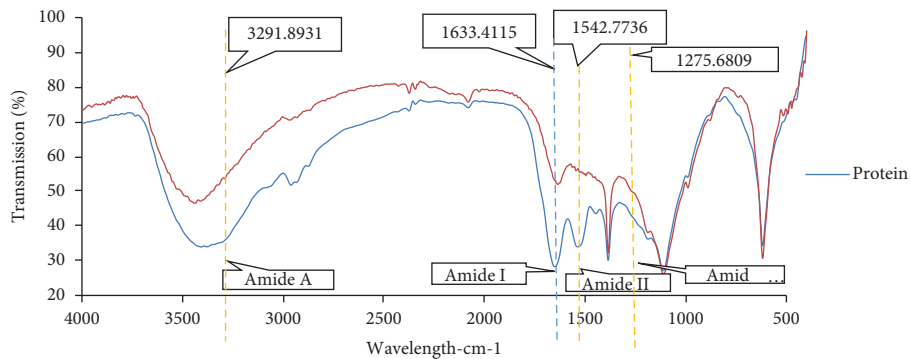
FIGURE 9: Biuret test for the extracted solutions.
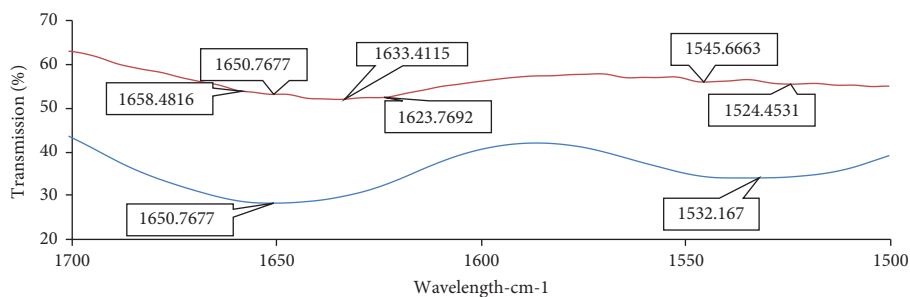

FIGURE 10: FT-IR of raw hoof and extracted keratin.

FIGURE 11: Expanded region (amide I and II) of the corresponding FTIR spectra.

3.7.3. Moisture Content. The amount of moisture in keratin powder has been measured in terms of moisture content. About 11.31% moisture content was achieved from the analysis. The value indicates that keratin material has better resistance to deterioration because of moisture. Researchers indicate that if the moisture content in the keratin material does not exceed 14% [28], it has better resistance to the moisture effect.

As discussed in the proximate analysis of the raw hoof, the moisture content in the raw hoof was about 5.96%. Larger moisture content was recorded for regenerated keratin than the raw hoof sample. This is because of the lower surface area of the keratin, which allows the hydrophilic site of keratin to react with water. Having a lower degree of crystallization in keratin than the raw material permits the formation of easier water bound to the hydrophilic group of keratins [45].

4. Conclusion

This study realizes that keratin can be extracted from slaughtered cattle hoofs using a solution of sodium hydroxide and urea.

(i) Around 75% protein content of the raw hoof has been a good indication of the material to be a good keratinous source.

(ii) Concentration of NaOH and temperature have the most significant effect on the quality and quantity of keratin.

(iii) The optimum condition for the extraction of keratin protein from cattle hoof was found to be 0.5 M NaOH and 60 minutes reaction time at 55°C temperature.

(iv) The FTIR spectra, indicates that amide I and amide II occur at a wave length of 1633 and 1542 cm^{-1} for raw cattle hoof and at wave length of 1650 and 1542 cm^{-1} for keratin protein, respectively.

(v) In conclusion, by employing optimum thermo-chemical conditions, the protein structure/quality can be maintained during extraction.

(vi) Further study will entail increasing the possibility of keratin extraction from cattle hoof by using other extraction methods such as reducing, steam explosion, and microwave methods.

Data Availability

All the data is included in the manuscript, however, any additional data can be provided upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

References

[1] T. K. Kumawat, A. Sharma, V. Sharma, and S. Chandra, *Keratin Waste: The Biodegradable Polymers*Intech Open, Keratin, 2018.

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

[3] V. Agarwal, A. G. Panicker, S. Indrakumar, and K. Chatterjee, "Comparative study of keratin extraction from human hair," *International Journal of Biological Macromolecules*, vol. 133, pp. 382–390, 2019.

[4] A. F. Ferreira, "Biorefinery concept," in *Biorefineries*, pp. 1–20, Springer, Cham, 2017.

[5] R. C. de Guzman, J. M. Saul, M. D. Ellenburg et al., "Bone regeneration with BMP-2 delivered from keratose scaffolds," *Biomaterials*, vol. 34, no. 6, pp. 1644–1656, 2013.

[6] P. Hill, H. Brantley, and M. Van Dyke, "Some properties of keratin biomaterials: kerateines," *Biomaterials*, vol. 31, no. 4, pp. 585–593, 2010.

[7] N. Eslahi, F. Dadashian, and N. H. Nejad, "An investigation on keratin extraction from wool and feather waste by enzymatic hydrolysis," *Preparative Biochemistry & Biotechnology*, vol. 43, no. 7, pp. 624–648, 2013.

[8] K. Kida, S. Morimura, J. Noda, Y. Nishida, T. Imai, and M. Otagiri, "Enzymatic hydrolysis of the horn and hoof of cow and buffalo," *Journal of Fermentation and Bioengineering*, vol. 80, no. 5, pp. 478–484, 1995.

[9] S. Feroz, N. Muhammad, J. Ratnayake, and G. Dias, "Keratin-Based materials for biomedical applications," *Bioactive Materials*, vol. 5, no. 3, pp. 496–509, 2020.

[10] A. Shavandi, T. H. Silva, A. A. Bekhit, and A. E. D. A. Bekhit, "Keratin: dissolution, extraction and biomedical application," *Biomaterials Science*, vol. 5, no. 9, pp. 1699–1735, 2017.

[11] Y. Ji, J. Chen, J. Lv, Z. Li, L. Xing, and S. Ding, "Extraction of keratin with ionic liquids from poultry feather," *Separation and Purification Technology*, vol. 132, pp. 577–583, 2014.

[12] Q. Q. Wang, L. Zhang, Q. Q. Wang, J. Liu, P. Zhu, and Z. N. Jiang, "Sodium sulphide extraction of human keratin," *Ferroelectrics*, vol. 529, no. 1, pp. 105–112, 2018.

[13] S. M. Martelli and J. B. Laurindo, "Chicken feather keratin films plasticized with polyethylene glycol," *International Journal of Polymeric Materials*, vol. 61, no. 1, pp. 17–29, 2012.

[14] V. Agarwal, N. Varghese, S. Dasgupta, A. K. Sood, and K. Chatterjee, "Engineering a 3D MoS2 foam using keratin exfoliated nanosheets," *Chemical Engineering Journal*, vol. 374, pp. 254–262, 2019.

[15] G. Rocha Plácido Moore, S. Maria Martelli, C. Gandolfo, P. José do Amaral Sobral, and J. Borges Laurindo, "Influence of the glycerol concentration on some physical properties of feather keratin films," *Food Hydrocolloids*, vol. 20, no. 7, pp. 975–982, 2006.

[16] S. Sharma and A. Gupta, "Sustainable management of keratin waste biomass: applications and future perspectives," *Brazilian Archives of Biology and Technology*, vol. 59, no. 0, 2016.

[17] A. E. Falaye and S. O. Sule, "Chemical composition of differently processed cattle hoof meal waste as feedstuff ingredient," *Ukrainian Journal of Veterinary and Agricultural Sciences*, vol. 3, no. 1, pp. 47–51, 2020.

[18] B. O. Alao, A. B. Falowo, A. Chulayo, and V. Muchenje, "The potential of animal by-products in food systems: production, prospects, and challenges," *Sustainability*, vol. 9, no. 7, p. 1089, 2017.

[19] M. J. Amiri, M. Bahrami, and F. Dehkhodaie, "Optimization of Hg (II) adsorption on bio-apatite based materials using CCD-RSM design: characterization and mechanism studies," *Journal of Water and Health*, vol. 17, no. 4, pp. 556–567, 2019.

[20] P. Kakkar, B. Madhan, and G. Shanmugam, "Extraction and characterization of keratin from bovine hoof: a potential material for biomedical applications," *SpringerPlus*, vol. 3, no. 1, p. 596, 2014.

[21] J. G. Rouse and M. E. Van Dyke, "A review of keratin-based biomaterials for biomedical applications," *Materials*, vol. 3, no. 2, pp. 999–1014, 2010.

[22] V. K. Thakur, M. K. Thakur, and M. R. Kessler, Eds., *Nanocomposites: Science and Fundamentals*, John Wiley & Sons, Vol. 7, , Hoboken, New Jersey, United States, 2017.

[23] Si Jr., "Development and characterization of keratin-based biofilm from bovine hoof waste for tissue engineering application," *Tissue Engineering Part a*, vol. 22, p. 140, 2016.

[24] N. B. Kamarudin, S. Sharma, A. Gupta, C. G. Kee, S. M. S. B. T. Chik, and R. Gupta, "Statistical investigation of extraction parameters of keratin from chicken feather using Design-Expert3," vol. 7, no. 2, pp. 127–129, 2017.

[25] M. Khumalo, B. Sithole, and T. Tesfaye, "Valorisation of waste chicken feathers: optimisation of keratin extraction from

waste chicken feathers by sodium bisulphite, sodium dodecyl sulphate and urea," *Journal of Environmental Management*, vol. 262, Article ID 110329, 2020.

[26] B. M. Assis, V. A. S. Vulcani, L. A. F. Silva et al., "Biochemical composition of the hoof capsule of buffaloes and its influence on hoof quality," *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, vol. 69, no. 1, pp. 57–64, 2017.

[27] G. T. Gindaba, S. G. Filate, and B. B. Etana, "Extraction and characterization of natural protein (deratin) from waste chicken feather," *International Journal of Modern Science and Technology*, vol. 4, no. 7, pp. 174–179, 2019.

[28] T. Tesfaye, B. B. Sithole, and D. Ramjugernath, "Preparation, characterization and application of keratin based green bio-films from waste chicken feathers," *International Journal of Chemical Sciences*, vol. 16, no. 3, 2018.

[29] 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 applied science and technology*, pp. 163–177, 2020.

[30] Aoac, "Official methods of analysis," *Assoc. Anal. Communities.*vol. 1, pp. 141–144, 2000.

[31] O. N. D. R. E. J. Krejci, P. A. V. E. L. Mokrejs, and S. Sukop, "Preparation and characterization of keratin hydrolysates," in *Proceedings of the Mathematical Methods and Techniques in Engineering and Environmentyal Science*, pp. 308–311, 2011 November.

[32] B. Chiego and H. Silver, "The effect of alkalis on the stability of keratins," *Journal of Investigative Dermatology*, vol. 5, no. 2, pp. 95–103, 1942.

[33] P. Das, P. P. Borah, and L. S. Badwaik, "Transformation of chicken feather keratin and pomelo peel pectin into biode-gradable composite film," *Journal of Polymers and the En-vironment*, vol. 26, no. 5, pp. 2120–2129, 2018.

[34] J. Barone and W. Schmidt, *Polymer Composites Containing Keratin*, 2006.

[35] Y. Nagai and T. Nishikawa, "Alkali solubilization of chicken feather keratin," *Agricultural and Biological Chemistry*, vol. 34, no. 1, pp. 16–22, 1970.

[36] M. I. Said, F. N. Yuliati, and M. Sukma, "The effects of acidic and alkaline hydrolysis process on some physical and chemical properties of broiler chicken feathers," *Iranian Journal of Applied Animal Science*, vol. 9, no. 3, pp. 529–540, 2019.

[37] S. S. Maier, H. Rajabinejad, and I. I. Buciscanu, "Practical ways of extracting keratin from keratinous wastes and by-products: a review," *Environmental Engineering and Management Journal*, vol. 15, no. 5, pp. 1131–1147, 2016.

[38] O. D. Fagbemi, B. Sithole, and T. Tesfaye, "Optimization of keratin protein extraction from waste chicken feathers using hybrid pre-treatment techniques," *Sustainable Chemistry and Pharmacy*, vol. 17, Article ID 100267, 2020.

[39] L. Li, S. Yang, T. Chen, L. Han, and G. Lian, "Investigation of pH effect on cationic solute binding to keratin and partition to hair," *International Journal of Cosmetic Science*, vol. 40, no. 1, pp. 93–102, 2018.

[40] A. Gupta, P. Kumar, R. Bin Mohd Yunus, and N. Binti Kamarudin, "Extraction of keratin protein from chicken feather," *Chemeca 2011: Engineering a Better World*, p. 2200, Sydney Hilton Hotel, NSW, Australia, 2011.

[41] M. Foggia, P. Taddei, A. Torreggiani, M. Dettin, and A. Tinti, "Self-assembling peptides for biomedical applications: IR and Raman spectroscopies for the study of secondary structure," *Proteomics Research Journal*, vol. 2, no. 3, p. 231, 2011.

[42] A. Aluigi, A. Varesano, A. Montarsolo et al., "Electrospinning of keratin/poly (ethylene oxide) blend nanofibers," *Journal of Applied Polymer Science*, vol. 104, no. 2, pp. 863–870, 2007.

[43] L. M. Wangatia and G. B. Tseghai, "Cationization of cotton using cattle hoof and horn for salt-free reactive dyeing," *Journal of the Textile Institute*, vol. 107, no. 11, pp. 1375–1380, 2016.

[44] S. Sharma, A. Gupta, A. Kumar, C. G. Kee, H. Kamyab, and S. M. Saufi, "An efficient conversion of waste feather keratin into ecofriendly bioplastic film," *Clean Technologies and Environmental Policy*, vol. 20, no. 10, pp. 2157–2167, 2018.

[45] X. Wang, Z. Shi, Q. Zhao, and Y. Yun, "Study on the structure and properties of biofunctional keratin from rabbit hair," *Materials*, vol. 14, no. 2, p. 379, 2021.