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Research Article

Optimization of Keratin Hydrolysate Extraction from Tannery Sheep Hair Waste

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Tannery hair wastes are becoming a challenge for tanners regarding environmental pollution control and human health. In this study, an experiment had been designed to hydrolyse sheep hair in an alkaline medium, and the operational condition for the alkaline extraction of KH has been modeled and optimized. The structure, morphology, functional groups, particle size, and molecular mass of the KH extracts were evaluated experimentally by scanning electron microscopy, X-ray diffraction, Fourier transform infrared spectroscopy (FTIR), particle size analysis, and SDS-PAGE analysis, respectively. FTIR analysis of the extract confirmed the presence of carboxylic, amide, and aldehyde functional groups and alkyl side chains of amino acids. The molecular weight of the extracted keratin ranges between 3–15 kDa, and X-ray diffraction (XRD) analysis showed an amorphous form of structure with two peaks at 2 theta of 9.36° and 21.16° due to α-helix and β- sheet structure in keratin. Response surface methodology (RSM) coupled with BOX-Behnken design was applied as a statistical tool to investigate the effect of extraction time, the concentration of the hydrolysing agent, and temperature on the response variable (yield of keratin protein). The concentration of the hydrolysing agent was found to be the most significant factor affecting the speed of extraction, but its gradual increase tends to affect the protein content of the extract. Optimum parameters of 0.5 N, 80°C, and 3.5 hr were obtained for the concentration of NaOH, temperature, and extraction time, respectively, with a maximum average protein yield of 91.5% and a percentage total nitrogen content of 14.6% using the Kjeldahl method and 86.57% using the biuret test method.

1. Introduction

Solid waste management in industrial society has become a recent interest due to the alarming effects caused by bad effluents and potential bio-compounds that can be extracted [1]. With developing urbanization, food industries, especially the meat market, slaughterhouses, and the leather industry, produce millions of keratinous biomass [2]. Nowadays, a large number of keratin byproducts have been wasted, which is a potential threat to the environment [2]. The management of keratin waste generated in the leather industry and slaughterhouses is a significant concern for many nations across the globe [3]. Due to its slow degradation, it stays in the dumps or waste streams for a long time, occupying large volumes of space. Over time, the accumulated hair increases the nitrogen concentration in the water bodies, causing problems for aquatic life [4–6]. Leather and textile production have the largest share in the generation of keratin-containing waste.

During the process of leather manufacturing, a substantial amount of solid and liquid waste is generated [7]. Some of the solid wastes generated are being value-added for practical use, but the majority of these wastes are dumped and become a source of pollution. Millions of tons of

keratinous waste are generated annually globally [8]. Ethiopian tanneries, for example, have an average daily soaking capacity of 107,850 pcs of sheep skin, 51,550 pcs of goat skin, and 9,800 pcs of hide [9], where, from the daily soaking capacity of 107,850 pcs of sheep skin about 40,000 kg (20–30%) of sheep hair waste is being generated and discharged as a solid waste [9, 10]. Conventionally, two methods of unhairing are being applied in the manufacture of leather [7, 10, 11]. The hair-burning method generates a significant amount of toxic and harmful sulfur-containing wastewater with COD higher than 10,000 mg/L. This accounts for about 40% of a total load of leather wastewater [12]. Therefore, the hair-saving unhairing method is the ultimate solution to lower the pollution from leather industries which may be realized by replacing the hair-burning unhairing method with the hair-saving unhairing method. However, nowadays most tanners prefer the hair-burning unhairing method due to the fear of a huge stack or deposit of hair once it is recovered, leading to increased cost. Thus, the use of hair as a resource may break the bottleneck in the wider application of the hair-saving unhairing method.

The substitution with the "hair-saving unhairing method" is an irresistible trend as people's consciousness of environmental protection is growing, environmental regulations are becoming more rigid, and the "hair-burning unhairing method" incurs severe environmental pollution. In addition to this, substitution will reduce the cost of wastewater treatment. Furthermore, if the problem of not using industrial hair waste as a resource is solved, hair will become another pivot for economic growth. Therefore, there is an urgent need in the field for a technical solution to the problem of the use of hair as a resource.

Keratins are fibrous sulfur-rich proteins with a molecular weight from 3 kDa up to 60 kDa. It is mainly found in two forms α and β-keratin. α-keratins are abundantly found in soft tissues such as sheep wool, skin, and hair. These are rich in cysteine and contain fewer amounts of hydroxyl proline and proline amino acids. However, β-keratins are present in the hard tissue protein of bird feathers, fish scales, nails, and others [2, 13], The keratin by-products have 90% of proteins, 15 to 18% nitrogen, 2 to 5% sulfur, 3.2% mineral elements, and 1.3% fat [2, 14]. Several difficulties exist in the production of keratin hydrolysates due to the nonreactivity and stability of keratin [15]. Several techniques have been investigated to extract keratin hydrolysates. Hydrothermal, acid, alkaline, and enzymatic hydrolysis are well-known techniques being used for keratin extraction. During hydrolysis, chemicals break both types of disulfide and peptide bonds in proteins, and as a result, the structure of keratin hydrolysate is changed [16]. As a result of the breakage of both types of bonds (disulfide and peptide), the resulting structure of keratin hydrolysates is different from the original structure of keratin protein [15].

Keratin present in tannery hair wastes has been utilized for several industrial applications such as leather and textile processing and agriculture input [2, 5, 17–19], the development of keratin-based biomaterials platform [2, 13], pharmaceutical and cosmetic application [17, 20–22]. Another study which was conducted by using sheep wool as raw material, alkaline as a hydrolysing agent, and temperatures of 75, 85, 95, and 99°C reported a percentage yield of 87% using 8% NaOH at 95°C [18]. However, the concentration reported as 8% NaOH is too much so the wool becomes a limiting reactant and excess NaOH remains in the final product, leading to another cost of HCl for pH adjustment. In Ethiopia, a study was conducted on white sheep hair waste using alkaline hydrolysis (1 N NaOH) with a yield of 87% at 80°C for 3 hrs was obtained [5]. In general, a lack of intensive optimization based on the specific raw material properties will end up with too much inorganic oxide in the final keratin hydrolysate extract. This has been reported to be the cause of effluent load during the application process in the leather retanning process as keratin filler [23].

Protein waste from various sources like poultry, slaughterhouses, the leather industry, and human hair is known to cause negative effects on humans and the environment [16]. From both an economic and environmental perspective, it is soundly desirable to develop an effective and profitable process to use waste resources. The best technique to address such problems is to develop systems that utilize waste material as a resource. The circular economy aims to extend material life and encourage recycling to maximize material services while reducing environmental impact and resource utilization [24]. Therefore, recycling of protein waste sources is very accessible, and developing a method for extracting keratin from such wastes was the objective of many studies due to their biodegradability and biocompatibility [5, 15, 16]. Even though protein waste could be used as a valuable resource and reduce environmental pollution, limited studies or even no studies were conducted on the extraction and characterization of keratin from Ethiopian mixed tannery sheep hair waste. Conducting extraction on mixed hair waste increases the production capacity and, furthermore, will help to investigate the effect of colour due to random mixing on the dyed crust during leather retanning and dyeing application. Therefore, this study aims to optimize the extraction process and characterize keratin hydrolysates from mixed sheep hair waste using alkaline hydrolysis methods.

2. Materials and Methods

2.1. Materials. Fresh sheepskins were procured from the Addis Ababa Abattoir in Addis Ababa, Ethiopia. Analytical grade chemicals and reagents used for laboratory experiments such as sodium hydroxide pellet (NaOH), 99.0%; potassium sodium tartrate, standard protein (BSA), 98%; potassium iodide (KI), 99.8%; hydrochloric acid 37%w/w (HCl); sulphuric acid (H_2SO_4), 98%; nitric acid (HNO_3), 65%; per chloric acid, 70%; sodium chloride (NaCl), 99.5%; boric acid, 99.5%; copper sulphate, 99%; and dichloromethane, 99.5%; they were obtained from Leather and Leather Products Industry Research and Development Center. Laboratory equipment and instruments mainly used in this study were round bottom flask, water bath, digital electronic balance, autoclave sterilizer, Soxhlet apparatus, digital pH meter, analytical weighting balance, Perkin Elmer UV/VIS spectrometer, hot air oven, testing equipment such

as Kjeldahl, FT-IR Spectroscopy Perkin Elmer, X-ray power Diffraction (XRD-X-ray tube cu40kv, 44 mA, Rugaku, Ultima IV), and scanning electron microscope (model JSM840A SEM microscope operating at 10 kV).

2.2. Methods. Sheep hair waste samples were collected using the composite method of sampling for the laboratory-scale extraction. The percentage composition of sheep hair based on the wet salted weight of skin was determined from a predetermined number of sheepskins procured from Addis Ababa Abattoir. The paint unhairing process was carried out in Model Tannery at Leather and Leather product Industry research and Development centre. Whereas sheep hair waste for pilot scale extraction was collected from Ethiopian tanneries (Ethiopia Tannery around Mojo and Elico in Addis Ababa) using the hair-saving method of hair removal. The experimental study was conducted at the Ethiopian Leather and Leather products Industry research and Development Center, which is located in the Akaki-KalitySub-city of Addis Ababa and some laboratory analysis work was carried out at the Central Laboratory of Addis Ababa, Science and Technology University (AASTU) and Adama Science and Technology University (ASTU).

2.2.1. Preparation of the Raw Material. The raw sheep hair sample was washed several times with sufficient excess warm water to remove sodium chloride (NaCl), sodium sulfide (Na_2S), and lime ($Ca(OH)_2$) that were used during preservation and paint unhairing, respectively. The washed sheep's hair samples were dried for about 48 hrs at 50°C. Degreasing was carried out using a mixture of sodium bicarbonate (1 g/L), ammonia (1 g/L), and an anionic degreasing agent (1 g/L) to remove fat on the surface of the hair; thereafter, the sample was thoroughly washed with distilled water to remove the remaining degreasing agents and rinsed with deionized water to avoid interference of degreasing agents during the hydrolysis reaction. Finally, the treated hair samples were cut into pieces to increase the surface area of contact with the hydrolysing agent and hence extraction efficiency.

2.2.2. Characterization of the Raw Material. The washed, treated, dried, and comminuted sheep hair samples were characterized for ash, nitrogen, and fat content. Ash is the inorganic residue obtained after the combustion of the keratin biomass. The total ash content of the treated hair samples was determined in triplicate, where 5 g of the treated hair sample was weighed and kept in a preweighed, clean, and dry crucible dish and heated on a heating mantle that was kept inside a cupboard hood until it ceased smoking. Then the dishes were placed in a muffle furnace and heated at 750°C for 3 hrs and finally, the total ash content of the hair samples was calculated in relation to the dry weight of the original sample after 3 hrs of sample ignition using equation (1). The nitrogen content was determined using the Kjeldahl Method. This method has been used in nitrogen determination and then multiplied by a conversion factor as determined by the following equation (2)to determine protein content. Fat content was determined in triplicate using the Soxhlet apparatus, where 10 g of the hair sample was taken for extraction using dichloromethane with an automated extraction Unit E-816 Soxhlet (BUchi), and the fat content was evaluated using the equation (3).

$$\%\text{Ash} = \frac{\text{weight of crucible \& ash} - \text{weight of the crucible}}{\text{weight of hair sample}} \times 100, \tag{1}$$

$$\text{Protein content}\,(\%) = \text{Nitrogen content in the sample} * 6.25, \tag{2}$$

$$\%\ \text{Fat content} = \frac{\text{Weight of extract \& reciever after drying} - \text{Weight of receiver}}{\text{Weight of sample}} x100. \tag{3}$$

2.2.3. Extraction of KH. A 20 g of prepared twenty samples were soaked in 0.25 L of the 0.5 N, 0.75 N, and 1 N NaOH solutions for 1.5, 2.5, and 3.5 hrs in a conical flask at 65, 80, and 95°C based on the designed experiment using the response surface methodology (RSM) approach coupled with the Box Behnken design. The conical flasks with the samples and solution of the hydrolysing agent inside were put in an autoclave for extraction. The extracted keratin hydrolysate (KH) was further purified by vacuum filtration. The hydrolysate was concentrated by evaporating in the water bath and centrifuged at 10,000 rpm for 5 minutes. The KH has been concentrated again in a water bath to make it ready for dialysis. All twenty samples were dialyzed using membrane dialysis for about 72 hrs. The dialysis products were further concentrated in a water bath, and then a 2M HCl (37%) solution was used to adjust the pH of the concentrated KH to the required pH of 4.2 then precipitated and centrifuged at 10,000 rpm for 5 minutes to form keratin powder.

2.2.4. Characterization of Extracted KH. The total ash content of the keratin hydrolysate powder was determined according to Society of leather Technologist and Chemists, SLC 6 [24, 25]. The total nitrogen content of the keratin hydrolysate powder was determined according to SLC 7 (IUC 10; BS 1309 : 7) using the Kjeldahl Method. The hydrodynamic radii of particles of KH were analysed using DLS with a high-performance particle size analyser

(Zetasizer Nano series, Malvern) at 25°C operating at 4 mW He-Ne laser powder with a scattering angle of 175°C and a wavelength of 633 nm. The percentage yields of protein for samples were evaluated using the Biuret test method and using the Kjeldahl method and multiplied by a constant of 6.25. The contents were cooled to room temperature, and the absorbance for each sample was measured. A graph of absorbance and concentration of the known standard protein and the unknown protein was developed and a general equation was developed using the following equation:

$$\mathbf{Y}(\%) = \frac{\mathbf{W_s}}{\mathbf{0.95W_{cw}}}\ \mathbf{x100}, \tag{4}$$

where Y is the percentage protein yield, W_s is the dry weight of extracted samples obtained from UV-visible spectroscopy analysis, and W_{cw} is the initial dry weight of the hair sample used for extraction. The extracted keratin protein was analysed with a UV-visible spectrophotometer and the absorbance of each sample at 540 nm was recorded to determine protein concentration (mg/ml) in the solution. The functional groups of the developed keratin product were analysed using an FTIR spectrophotometer (SHIMADZU IR Affinity-1S). The spectrum was obtained in a transmission mode under a scanning rate and a resolution of 32 times/min and 4 cm^{-1}, respectively.

The crystal structure and chemical composition of the keratin powder were characterized using the X-ray power diffraction (XRD), a nondestructive analytical technique. The machine used for the analysis was an X-ray power diffraction instrument (XRD-X-ray tube cu40kv, 44 mA, Rugaku, Ultima IV). The analysis was undergone at a scan speed of 6 rev/min over an angle range of 5° to 80° with the power set to 40 kV and 44 mA. The surface morphologies, formation of porosity, and aggregates that were expected on the powder of keratin from sheep hair hydrolysis were determined by scanning electron microscopy (SEM, model JSM840A).

2.3. Experimental Design. Box-Behnken experimental design was performed during the study period with the three selected process variables at the three levels as shown in Table 1. The range of variables was selected from the previously published articles and by conducting preliminary laboratory experiments. The experimental design of the study was expressed as a 3^3 which is anticipated to generate 27 runs, however, the number of experimental runs was reduced to 20 runs using Design Expert 7.0. The benefit of the Box-Behnken design approach is to evaluate interactions among the experimental variables and their impacts on the response of KH extraction (protein yield%). The combination of each variable with a lower and higher level of the run was done randomly. The interaction effects of three variables and their influences on the response of KH extraction (protein yield%) were studied.

To check the adequacy, the variance analysis model (ANOVA) was used. The model used to fit the results of the three-level design is represented by desired responses from the following equation:

Table 1: The Box-Behnken experimental design of KH extraction.

Variables	Low (−)	Middle (0)	High (+)
Temperature (°C)	65	80	95
The concentration of NaOH (N)	0.5	0.75	1.0
Time (hr.)	1.50	2.50	3.50

$$\mathbf{Y} = \mathbf{b_o} + \sum_{\mathbf{i=1}}^{\mathbf{n}} \mathbf{b_i X_i} + \sum_{\mathbf{i=1}}^{\mathbf{n}} \mathbf{b_{ii} X_i^2} + \sum_{\mathbf{i=1}}^{\mathbf{n-1}} \sum_{\mathbf{j=2}}^{\mathbf{n}} \mathbf{b_{ij} X_i X_j} + \boldsymbol{\varepsilon}, \tag{5}$$

where, $X_1X_2, \ldots, X_n$ is the input factors that can influence the response Y; n is the number of variables, b_o is the constant intercept, b_{ii} ($i = 1, 2, \ldots, n$) is the quadratic coefficient, b_{ij} ($i = 1, 2, \ldots, n$; $j = 1, 2, \ldots, n$) is the interaction of the coefficient, and ε represents the random error.

3. Results and Discussions

3.1. Characterization of the Raw Material

3.1.1. Fat Content. The average fat content of the triplicate samples was evaluated to be 5.20% (Table 2) before degreasing action which is a little bit less than the one reported by [23] (8.2%). This might be due to the difference in the breed of sheep and their feeding system. However, the result was in agreement with the one published by [26]. The fat content after degreasing was reduced to a lower level of 2.69% in which an average of 48% fat removal was carried out which is important to control the softening action of the extracted keratin hydrolysate (KH) during its application in leather processing based on the type of article to be produced.

3.1.2. Ash Content. Triplicate samples were analysed and it was observed that the ash content was found to be an average of 12.73% of the sample weight as presented in Table 3which is in agreement with the one reported by the researcher [18]. However, it is far greater than the one reported in [23], namely, 2.3% from sheep wool. Ash content of the raw sheep hair is an indication of presence of inorganic oxide in the original material. This ash content was far lower than the one obtained from keratin hydrolysate due the fact that inorganic oxides used as hydrolysing agent also contributed to the ash content. So, too much ash is an indication of excessive dosage of the hydrolysing agent.

The ash content of the keratin extracts needs to be taken into consideration while developing an eco-benign retanning agent. Nowadays, manufacturers of leather chemicals are all under the pressure of developing retanning agents with low salt content. In this specific study, ash content of the developed KH is around 12.73% w/w which is a little bit higher than the one reported by [27], 10%, and almost in agreement with the one reported by [18] which is 12.16 ± 1.42, whereas ash content of most commercial retanning agents falls under the range of 30–40% w/w [27]. Therefore, the developed product is rich in organic materials that escaped during the process of determining the ash content and has relatively less hazardous inorganic oxides

Table 2: Fat and ash content of raw and treated sheep hair waste.

Trial no		1	2	3	Mean ± SD
Fat content	Before degreasing	5.52	5.22	4.87	5.20 ± 0.3
	After degreasing	3.06	2.87	2.14	2.69 ± 0.5
Ash content		12.8	12.72	12.68	12.73 ± 0.1
Nitrogen content		14.98	15.12	15.12	15.09 ± 0.1

Table 3: Total nitrogen and protein content determination using the Kjeldahl method.

Run no	0.1 NHCl consumed average ± SD	% Nitrogen content average ± SD	% Protein content average ± SD
	Acid titration to determine nitrogen		
1	101.5 ± 2.0	14.2 ± 0.3	88.9 ± 7.2
2	98.8 ± 2.6	13.8 ± 0.4	86.6 ± 2.2
3	97.3 ± 2.5	13.6 ± 0.3	85.2 ± 1.9
4	95.2 ± 2.8	13.3 ± 0.6	83.3 ± 3.5
5	100.0 ± 2.5	14.0 ± 1.5	87.5 ± 9.1
6	81.3 ± 2.9	11.4 ± 1.4	71.2 ± 8.8
7	96.2 ± 2.8	13.5 ± 0.7	84.2 ± 4.6
8	104.5 ± 1.0	14.6 ± 0.4	91.5 ± 2.3
9	99.8 ± 1.9	14.0 ± 0.2	87.4 ± 1.5
10	98.0 ± 2.2	13.7 ± 0.4	85.8 ± 2.6
11	99.8 ± 2.9	14.0 ± 0.5	87.4 ± 2.8
12	102.2 ± 2.5	14.3 ± 0.7	89.4 ± 4.16
13	93.7 ± 2.8	13.1 ± 0.7	82.0 ± 4.4
14	98.5 ± 3.0	13.8 ± 1.0	86.2 ± 6.5
15	86.3 ± 1.0	12.1 ± 0.4	75.6 ± 2.5
16	91.7 ± 1.8	12.8 ± 0.8	80.3 ± 5.2
17	101.2 ± 2.5	14.2 ± 0.4	88.6 ± 2.3
18	102.3 ± 1.8	14.3 ± 0.6	89.6 ± 3.7
19	92.7 ± 6.7	13.0 ± 1.0	81.2 ± 6.3
20	96.8 ± 2.6	13.6 ± 0.4	84.8 ± 2.3

and salinity compared with most commercial retanning agents. Furthermore, the relatively higher ash content of keratin hydrolysate compared with those obtained by the stated authors was caused by the alkaline medium of the hydrolysis reaction with optimum value of concentration at the lower border; thus, in order to obtain cleaner organic keratin hydrolysate for some industrial applications, further optimization of the concentration of NaOH below 0.5 N and repeated dialysis of the extract are necessary [23].

3.1.3. Nitrogen Content. The nitrogen content of the sheep hair sample was determined using the Kjeldahl method where 1 g of treated hair sample was taken in a triplicate, digested using the prepared acid mix to generate ammonium, as shown in Figure 1, which was further treated with alkaline (50% sodium hydroxide) solution to change it to ammonia, and then was distilled using steam at high temperature. The escaped ammonia due to steam distillation was condensed and trapped using boric acid, the amount of which was determined by titration, and the average was calculated to be 15.09% based on the dry weight of the hair sample, where the same study was conducted with a similar result by [21] and is higher than the value reported by [28], 14.56% and [23], 12.2%. Here, the protein content was estimated according to the analysis of total Kjeldahl nitrogen (TKN), which is obtained by multiplying the nitrogen content by a variable factor for each protein. Regarding keratin, the protein yield was calculated by multiplying the nitrogen content by a factor of 6.25 [22].

3.2. Extraction and KH Analysis. Keratin hydrolysate of different colours was obtained as shown in Figure 2. The result showed that the colour of the extract was derived from the pigment of the hair. The KH extracted was found to have a colour matching all the colours of sheep hair mixed randomly. Residues of the extraction were observed settling at the bottom and varying in amount from one run to another due to differences in the degree of hydrolysis.

It was observed that the alkaline hydrolysing agent solubilizes the sheep's hair sample, with the rate of solubilisation directly proportional to the concentration of hydrolysing agent and temperature. The amount of residue generated (8.9–27.3% (w/w)) was observed to be dependent on the values of independent variables selected from the designed experiment and shown in Table 4. The absorbance value of the extracted product at 540 nm around the optimum parameters was found to be high, which is an indication of the existence of more peptide bonds. However, for samples subjected to larger concentration of the hydrolysing agent and higher temperature, lower absorbance value resulted due to further breakage of the protein to

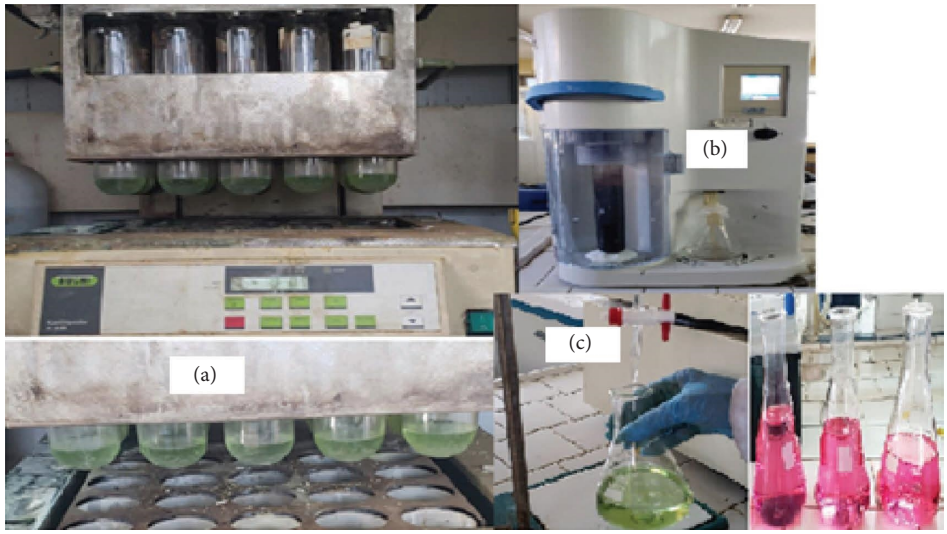


Figure 1: Determination of total nitrogen content (a) digestion, (b) distillation, and (c) titration.

(a) (b) (c) (d)

Figure 2: Extraction products: (a) white sheep hair, (b) red sheep hair, (c) mixed sheep hair, and (d) powder KH.

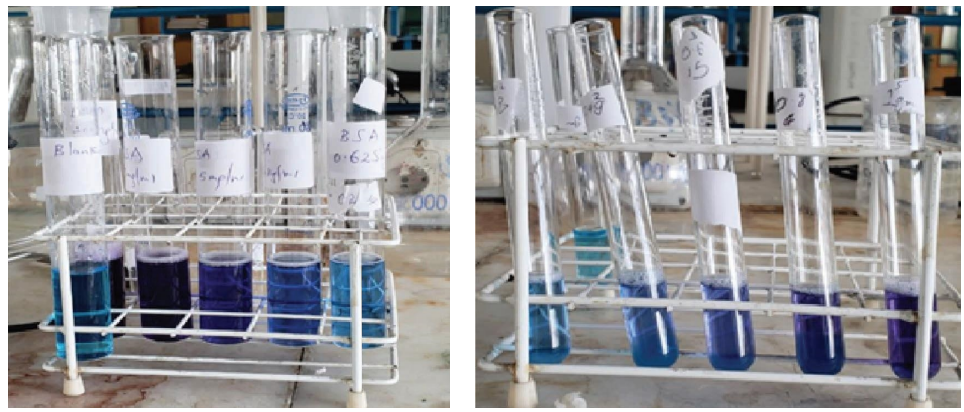
amino acid level. Here, due to the unreacted inorganic oxide used as a hydrolysing agent, the ash content of the extract was seen to have larger value. The reduction of protein to the amino acids level will end up with reduction in absorbance value while detecting using ultraviolet-visible spectroscopy due to the fact that UV-visible spectroscopy detects only the protein component with peptide bond. The Kjeldahl method of analysis detects both proteins forming chains using peptide bonds and those further reduced to amino acid levels. So, most of the time, the protein content analysed using the Kjeldahl method was observed to be larger than that determined using the biuret test method.

3.2.1. Protein Analysis. The addition of the Biuret reagent to the BSA standard protein (Bovine Serum Albumin) and the keratin extract turns sample solutions into purple colour, indicating the presence of peptide bonds in the BSA and keratin extract [29]. It was observed that the more the amount of BSA and keratin extract dissolved in the biuret reagent per unit volume of solution, the higher the intensity of the purple colour, as shown in Figure 3.

In addition to the qualitative analysis of determining the amount of peptide bond in the KH extract by observing the intensity of the purple color, and UV-visible spectrometer was also used to quantitatively determine the absorbance at

Table 4: Analysis result for percentage generation of residue and extract after centrifuging.

S/no	Weight plastic container (gm.)	Weight of container + dried residue (gm.)	Dried residue (gm.) of 3.35 gm sample	Percentage generation of residue (%)
1	12.649	11.9065	0.7425	22.164
2	13.391	12.5348	0.8562	25.558
3	12.927	12.6288	0.2982	8.9000
4	12.712	12.3338	0.3782	10.2900
5	12.505	13.412	0.7735	23.090
6	12.551	11.6364	0.9146	27.300
7	12.408	11.6619	0.7461	22.272
8	12.542	12.0679	0.4741	14.152
9	13.101	12.3811	0.7199	21.490
10	12.213	11.4241	0.7889	23.549
11	13.194	12.5621	0.6319	18.863
12	12.675	11.9137	0.7613	22.725
13	12.989	13.823	0.6997	20.887
14	12.852	12.4728	0.3792	11.319
15	12.613	11.8292	0.7838	23.397
16	12.554	116849	0.8691	25.943
17	12.661	11.9311	0.7299	21.788
18	12.701	11945	0.756	22.570
19	12.651	11.862	0.789	23.552
20	12.323	11.466	0.857	25.579



(a) (b)

Figure 3: Standard solution preparations for curve development (a) 10–0.625 mg/ml and (b) 0.2–1 mg/ml.

540 nm of each sample synthesized under different working conditions [29]. This helps to determine the concentration of keratin protein based on the amount of peptide bond and hence the percentage yield using the biuret test method.

Keratin hydrolysate extracts from different extraction conditions were taken for testing using the biuret test method, where an equal amount of the samples were dissolved in the biuret reagent of the same amount and it was observed that extracts from the optimum parameters of 80°C, 0.5 N, and 3.5 hr, as shown in Figure 4 was seen to have the purple colour of higher intensity. This colour of higher intensity is an indication that the sample has a higher number of peptide bonds [15, 30]. The intensity of the colour drops with temperature and concentration of hydrolysing agent (65°C to 95°C and 0.5 N to 1 N) due to the vigorous reaction and further breakage of the peptide bond and peptide molecule at the amino acid level. Furthermore, excessive utilization of the hydrolysing agent makes the product to saturate with unreacted inorganic oxide. On the other hand, at far lower temperatures and higher concentration of hydrolysing agent than the optimum values selected, a less intense purple colour appeared, indicating that there was insufficient temperature to degrade the keratin polypeptide to lower molecular peptide molecules. Two commercial protein fillers being used at the retanning stage of leather processing (celatan F and filler R) were compared with the KH extract, and the result showed that the extracted KH protein has more peptide bonds, as shown in Figure 5. The intensity of the purple colour for KH extract is comparable with that of the standard protein bovine serum albumin (BSA) and more intense compared with that of commercial protein fillers [31].

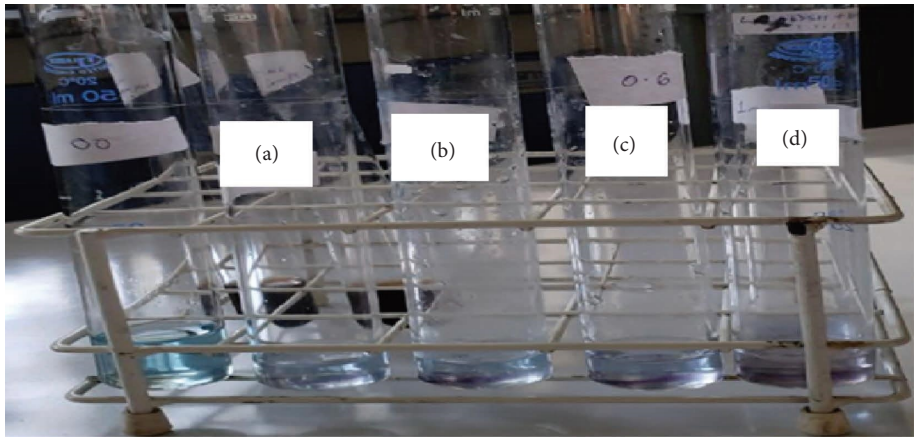


Figure 4: Biuret test for KH extract (a) 95°C, 1 N & 1.5 hr (b) 65°C, 1 N and 3.5 hr (c) 80°C, 0.5 N and 1.5 hr (d) 80°C, 0.5 N and 3.5 hr.

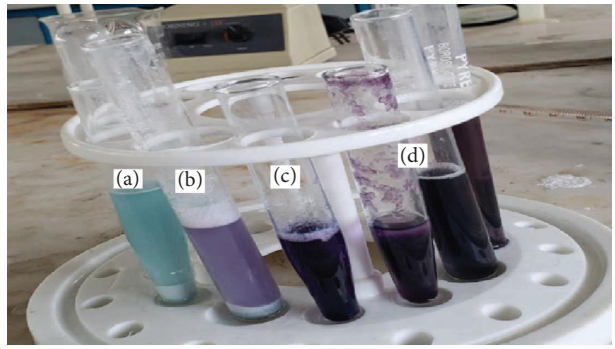


Figure 5: Biuret test method to compare KH with commercial protein fillers (a) filler R, (b) celatan F, (c) bovine serum albumin (BSA), and (d) keratin proteins.

3.2.2. UV-Visible Spectroscopy Analysis. Keratin solution exhibited different peaks over a wide range of wavelengths from 200–800 nm. Where, most of the peaks lay between 220 and 380 nm. The maximum peaks were observed at 260 and 280 nm due to the aromatic ring portion of amino acid groups. Peaks range from 220 to 260 due to amino acids and carboxylic acid groups forming a peptide bond [32]. In this specific study, the extracted keratin protein was analysed with a UV-visible spectrophotometer and the absorbance of each sample at 540 nm was recorded. The concentration (mg/ml) of protein in the solution was determined using the developed standard equation "$y = 0.0934x + 0.0141$," as shown in Table 5.

3.2.3. Development of a Standard Graph and Protein Yield Calculation. The absorbance value at 540 nm for the bovine serum albumin (BSA) of predetermined concentrations was analysed using UV-visible spectroscopy, which was used to develop a standard curve to determine the concentration of unknown samples, as shown in Figure 6. The best-fitting linear equation was generated to estimate the protein concentration for the unknown samples as $y = 0.0934x + 0.0141$, where "y" is the absorbance at 540 nm and "x" is the concentration of protein in the unknown sample in mg/ml. The value of $R^2 > 0.99$ indicates the strength of the linear relationship between the UV-visible spectroscopy absorbance of the sample protein and concentration which showed that the curve fitting to develop a linear equation relating the absorbance with concentration helps to determine the concentration of the unknown samples with great reliability.

3.3. Characterization Result for the Extracted KH

3.3.1. Ash Content. It was observed that the ash content was found to be inversely proportional to the percentage protein yield. The ash content of the extracted KH around the optimum parameters is lesser and it increases as the concentration of hydrolysing agent increases further away from the point of optimum parameters. This is an indication that ash content is directly proportional to the unreacted inorganic oxide impurities used during the hydrolysis reaction. The percentage of ash content analysed was in the range of 9% and 21.45% which is in agreement with the result obtained by [18]. An increase in the ash content of the keratin hydrolysate compared to the original raw sheep hair waste is due to inorganic oxides used for the hydrolysis reaction. So, larger the ash content depicts the excessive usage of NaOH (hydrolysing agent).

Ash content refers to the undesired inorganic oxides and salts, which may be derived from the reagents used for extraction purposes or inorganic oxides that originally exist

Table 5: Percentage particle size distribution for KH at different conditions by the intensity.

	Trial	a	b	c	d	e
Peak 1	Mean size ± SD (d.nm)	786.5 ± 299	2475 ± 686	1125 ± 224	1206 ± 167	877.4 ± 124
	Intensity (%)	91.9	87.1	39.1	68.5	62.1
Peak 2	Mean size ± SD (d.nm)	3.25 ± 0.71	0.70 ± 0.08	193.3 ± 41.4	246 ± 31	267.0 ± 35
	Intensity (%)	8.1	12.9	36.8	31.5	37.9
Peak 3	Mean size ± SD (d.nm)	0.00	0.00	0.00	0.00	0.00
	Intensity (%)	0	0	0	0	0

Where extraction condition: (a) at 0.5 N, 80°C, and 3.5 hr; (b) at 1 N, 95°C, and 1.5 hr; (c) at 0.5 N, 95°C, and 2.5 hr; (d) at 1 N, 95°C, and 2.5 hr; (e) at 1 N, 65°C, and 3.5 hr.

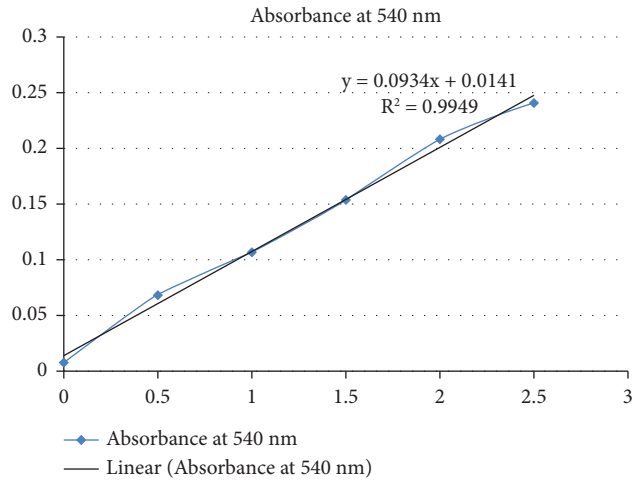


Figure 6: Standard curve to determine the concentration of unknown samples.

in the raw material [23]. Therefore, the higher percentage ash content usually has the implication of higher impurities or inorganic residue, which is most probably the unreacted alkaline oxides in the extract and salts generated during the reaction processes. The higher the ash content the lesser the amount of organic active components participating in the reaction during leather process application. This has an indication that ash content determines the greenness of the keratin extract, since it is an important feature of any retanning agent being produced and used in leather processing. Furthermore, it is a well-known fact that treatment of wastewater containing salinity load is one of the major problems associated with leather industry and retanning agents such as KH are significantly contributing to this salinity load. In general, the higher the percentage of ash content the higher the amount of unreacted oxides and other salts generated, which implies the less the amount of organic active component and the more effluent load generation while using the KH as a retanning agent.

3.3.2. Nitrogen Content. Determining the nitrogen content of the KH provides information on the extent of breakage of peptide bonds by hydrolysis reactions forming low molecular proteins and peptides, and on the amino acid composition of the sheep hair which confirms the transformation of the amino acid cysteine to cysteic acid following extraction. From the total nitrogen content determination, it was observed that higher concentrations and temperatures beyond 0.5 N and 80°C resulted in a decrease in the protein content of the KH extract. Table 3 shows the HCl consumption, total nitrogen, and percentage protein content determined using the Kjeldahl method in a triplicate run. The results were similar to those studied by [33, 34].

3.3.3. Particle Size Estimation for KH. From the DLS analysis result, it was observed that smaller particle sizes of 0.6912 nm (12.9%) at 95°C, 1 N, and 1.5 hr were detected compared to the extraction condition of 95°C, 1 N, and 2.5 hr which has the same concentration of hydrolysing agent and temperature of hydrolysis which is due to the short duration of hydrolysis giving the higher intensity of large-sized particles (2,475 nm) and is far larger compared with that of run with 2.5 hr (1206 nm). It was also observed that an increase in the concentration of the hydrolysing agent increases the intensity of large-sized particles, as presented in Table 5. The results obtained are better than the KH particle size obtained by [34] −3,838 nm and almost around the same size (better) than the particle size reported by [35] which is 1,516 nm. So, based on the observed result of KH extract since particle size and molecular weight distribution are core factors determining the ease of penetration of keratin fillers in the collagen matrix and sequence of application of chemicals, parameters resulting in KH extract with the majority of smaller particle size distribution are preferred for

mass production. Using a 0.5 N hydrolysing agent was observed to extract KH proteins with lower particle size and a temperature of 80°C produces smaller-sized extracts than 95°C.

3.3.4. SDS-PAGE Analysis. The analysis showed that the molecular weight is distributed throughout the band, and some fragments had molecular weight exceeding 20 kDa for keratin produced at 80°C. But the majority had a molecular weight ranging from 5.8 to 12 kDa which is similar to the study [36, 37]. The finding indicates that its molecular weight is lower compared to the molecular weight of most commercial protein fillers implicating that it has a higher tendency of penetrating through the collagen structure and filling interfibrillar spaces. Especially the smaller molecular mass of keratin is in agreement with that of the experimental process of applying keratin after chrome tanning because applying larger molecular-sized chemicals before that smaller molecular-sized one will help the smaller to penetrate through the void generated by the larger molecular-sized chemicals like chromium.

3.3.5. Fourier Transform Infrared Analysis. As shown in Figure 7, the relatively broad peak in the region of 3250–3300 cm^{-1} corresponds to the hydrogen-bonded -N-H and -O-H stretching motion of the amide functionality and absorbed water. The relatively less intense peak observed in the region of 2900–3100 cm^{-1} corresponds to the stretching motions of -C-H and -N-H groups. The peak observed in the region of 1400–1700 cm^{-1} is due to the carbonyl group of amide functionality.

However, the intense sharp peak at 1647.37 cm^{-1} is due to the stretching vibration of the amide carbonyl (-C=O) functional group. The stretching vibration of -C-N- and -C-C- functional groups and bending vibrations of the N–H functional group in–CNH were observed at 1237.03 cm^{-1}. The peak displayed at 1400.52 cm^{-1} was due to the bending vibration of the -CH_2 functional group, and a less intense peak at 1541.11 cm^{-1} correspond to the -C-N-H group bending vibration. The less intense sharp peaks between 1200 and 910 cm^{-1} are due to -C-N- group stretching vibration. The presence of characteristic peaks like amide -N-H, -C=O, -C-N-, and –CNH functionalities in this FT-IR pattern confirms the existence of building block amino acids forming peptide groups of keratin protein. The FTIR pattern of keratin extract in transmittance mode is expected to have peaks due to the characteristic structure and specific motion of important functional groups in its molecular structures like -CO–NH-, -NH_2, –CNH, and -C-H [27, 35].

3.3.6. XRD Analysis. The X-ray diffraction pattern showed that the keratin extracted has the general nature of an amorphous structural form instead of the crystalline form (Figure 8). However, there are two special places on (2 theta) where the crystalline nature of the keratin is displayed due to the crystalline nature of α-helix and β-sheet structures. It was observed from the XRD pattern that the two crystal structures had diffraction peaks of different intensities. The more intense and less broad peak α-helix appeared at 2 theta (9.36°), and on the other hand, the short and broader peak was observed at 21.16° corresponding β-sheet structures [37].

3.3.7. Scanning Electron Microscope (SEM) Analysis. The alkaline hydrolysis of the KH extract from tannery sheep hair waste has the morphologies using SEM at different resolutions of x170 and x300 as shown in Figure 9.

A highly porous structure was displayed on the surface as a morphological characteristic with a number of features. In the image at a resolution of x170 the keratin seemed to be small particles in aggregate form with observable porosity; however, as the resolution increases to x300 the crack and porosity were observed to be higher. The keratin powders underwent dramatic changes in shape and did not display the residue of a sheep hair-like structure, indicating that it have lost the same compact structural nature as sheep hair. The morphological view has the same appearance as the one extracted at 80°C and reported by [37–39], however, it is darker in colour and highly porous with more void spaces.

3.4. The Optimization Process of KH Extraction. The protein yield and concentration values are shown in Table 6. It was observed that all the three-selected parameters have their role in the cleavage of peptide and disulfide bond to some limited values beyond which a decrease or increase in value either has no effect at all or will harm the protein content. As the temperature increased from 65°C to 80°C an increase in protein yield was observed till 78°C–80°C, and beyond this temperature, the percentage of protein yield dropped gradually. A decrease in the concentration of the hydrolysing agent from 1 N to 0.5 N increases yield and the reverse is also true when the reaction condition is vigorous and the extract is subjected to a loss of its structure and even all its proteins might be reduced to an amino acid level. So the amount of keratin protein yield depends on the optimality of the parameters affecting the degree of degradation of the keratin biomass.

3.5. Statistical Analysis

3.5.1. Fit Summary. The output of different model summary statistics is shown in Table 7 and focused on the nonaliased model maximizing, the adjusted R^2 and predicted R^2 values being large, and additional terms being significant. Therefore, the quadratic model was suggested.

3.5.2. Analysis of Variance (ANOVA). The percentage yield of protein was fed to the software as a response to the selected design layout and model generation; analysis of variance (ANOVA) as shown in Table 7 and model fitness (Table 8).

The model found relevant for variance analysis was the quadratic regression model, where the model F value of 378.42 implies that 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 0.05 indicates that model terms are significant, where A, B, C, AB, AC, BC, A^2, and B^2 are

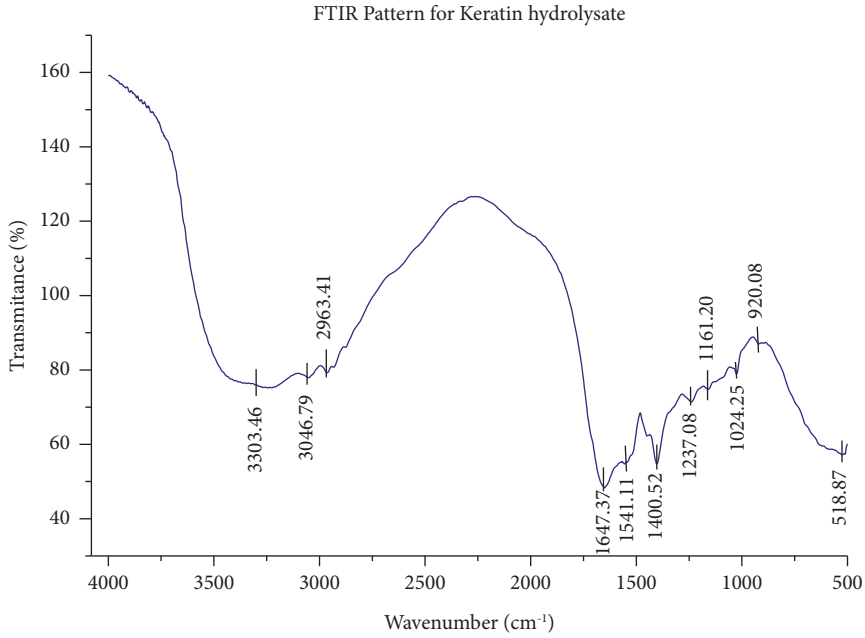


Figure 7: FTIR pattern for the extracted KH.

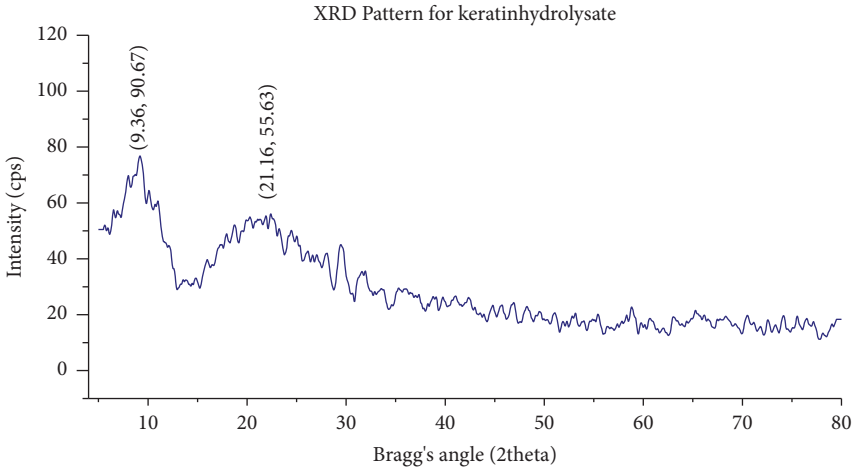


Figure 8: XRD analysis pattern of keratin hydrolysate.

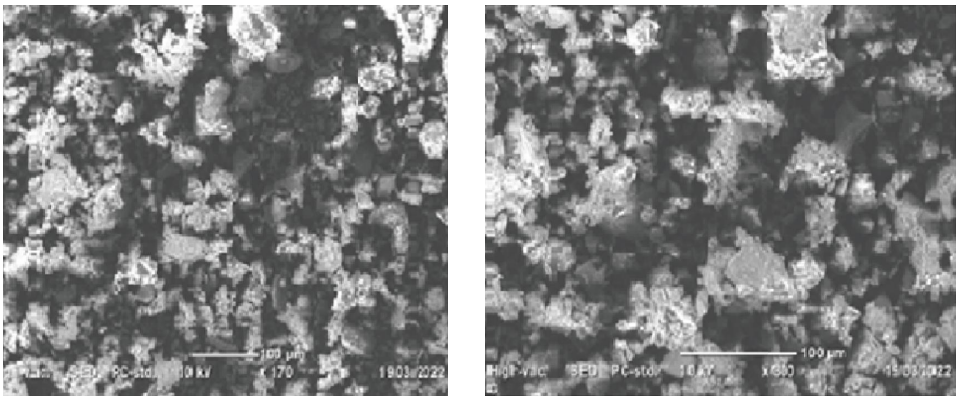


Figure 9: SEM image for the KH product at x170 and x300 resolution.

Table 6: Experimental responses of the predicted values.

Run no	Temperature (°C)	Concentration (*N*)	Time (hr.)	Absorbance at 540 nm	Protein yield (%)
1	80	0.75	2.5	0.791	82.7
2	65	0.50	1.5	0.746	80.1
3	80	1.00	3.5	0.744	78.9
4	95	1.00	2.5	0.682	74.3
5	65	0.50	3.5	0.803	82.9
6	65	1.00	2.5	0.696	74.4
7	95	0.75	2.5	0.731	77.5
8	80	0.50	3.5	0.821	86.6
9	95	0.50	2.5	0.761	79.2
10	80	0.75	1.5	0.779	82.1
11	80	0.75	2.5	0.787	83.6
12	80	0.50	1.5	0.786	83.3
13	65	0.75	2.5	0.748	78.1
14	95	0.75	3.5	0.719	78.2
15	95	1.00	1.5	0.676	74.0
16	95	0.75	1.5	0.714	77.6
17	65	0.75	1.5	0.761	78.1
18	95	0.50	2.5	0.758	81.8
19	80	1.00	1.5	0.742	78.8
20	65	1.00	3.5	0.712	73.6

Table 7: Fit summaries.

Source	Sequential p value	Adjusted R^2	Predicted R^2	
Linear	0.0174	0.3586	0.1627	
2FI	0.3486	0.3818	−0.0159	
Quadratic	<0.0001	0.9930	0.9829	Suggested
Cubic	0.4557	0.9942		Aliased

Table 8: Analysis of variance (ANOVA).

Source	Sum of squares	Df	Mean square	F value	p value	
Model	243.75	8	30.47	370.83	<0.0001	Significant
A	107.65	1	107.65	1310.12	<0.0001	
B	2.06	1	2.06	25.07	0.0004	
C	4.69	1	4.69	57.08	<0.0001	
AB	1.44	1	1.44	17.54	0.0015	
AC	8.50	1	8.50	103.42	<0.0001	
BC	0.9345	1	0.9345	11.37	0.0062	
A^2	1.02	1	1.02	12.43	0.0048	
B^2	97.84	1	97.84	1190.72	<0.0001	
Residual	0.9038	11	0.0822			
Cor total	244.66	19				

where A denotes concentration, B denotes temperature, and C denotes time. The model found relevant for variance analysis was the quadratic regression model, where the model F value of 378.42 implies that the model is significant. *P* values less than 0.05 indicate that model terms are significant, wherein in this case, A, B, C, AB, AC, BC, A2, and B2 are significant model terms.

significant model terms. Values greater than 0.100 indicate that the model terms are not significant, whereas the model term C^2 is not significant. This implies that the concentration of the hydrolysing agent, the temperature of extraction, the duration of extraction, the linear interaction between concentration and temperature, linear interaction between the concentration of the hydrolysing agent and the duration of hydrolysis, linear interaction between temperature and duration of hydrolysis, and the quadratic interaction effect between the concentration of the hydrolysing agent and temperature all significantly affect the KH protein yield.

The coefficient of regression (R^2) indicates the relationship between the experimental data and the expected response variable quantitatively. The predicted R^2 of 0.9855 is in reasonable agreement with the adjusted R^2 of 0.9936 which means the difference is less than 0.2. A signal-to-noise ratio of 69.1312 indicates an adequate signal, so the model can be used to navigate the design space. Where the coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant and the intercept in an orthogonal design is the overall average

response of all the runs. VIFs greater than 1 indicated greater multicollinearity; the higher the VIF, the more severe the correlation of factors.

3.5.3. Empirical Model. An empirical model for maximum yield of keratin protein in terms of the process parameters using coded factors was developed by the Box-Behnken Design experiment, where quadratic model equations in equation (6) were fitted to the data model for predicting the response variable, yield of keratin protein, in terms of coded and actual factors, respectively. The equation in terms of coded and actual factors can be used to make predictions about the response for given levels of each factor and is also fully used for identifying the relative impact of the factors by comparing the factor coefficients.

$$\begin{aligned}\%\ \text{Protein yield} &= 82.6 - 3.0A - 0.4B + 0.6C \\ &+0.4AB - 1.0\text{AC} + 0.4\text{BC} - 0.5\text{A}^2 - 4.8\text{B}^2.\end{aligned} \quad (6)$$

All the stated parameters have a significant effect on protein yield as p value for all is less than 0.05 ($P < 0.05$). The model equation describes how the protein yield was affected by individual variables by their (linear and quadratic) terms or double interaction. Negative coefficients indicate that factors negatively affect the yield. In this specific study, the double interaction effect of concentration and time and the quadratic effect of concentration and temperature affect the yield negatively.

3.5.4. Adequacy Check for the Developed Model. After investigating the effect of each independent variable individually and the interaction effect between each variable to obtain maximum protein yield analysed using ANOVA the next step was to check the adequacy of the model. The quality of model fit for the alkaline hydrolysis process was evaluated using Fisher's test (F-value), the probability value (p value), the lack of fit, the coefficient of determination (R^2), adjusted R^2 (R^2adj), and predicted R^2 (R^2pred). Where, the F value and p value of the quadratic model were found to be 378.42 and 0.0001, respectively, which implies that the quadratic model is significant. So the model can sufficiently predict the percentage yield of protein.

3.6. Interaction Effect of Variables on Protein Yield

3.6.1. Temperature and NaOH Concentration. The combined effect of temperature and concentration on the percentage of protein yield at a constant time of extraction (3.5 hr) is shown in (Figures 10(a) and 10(b)). It can be easily observed that protein yield increases with a decrease in concentration from 1 N to 0.5 N, and at the same time, an increase in temperature from 65°C to 80°C increases the percentage yield of keratin protein. However, a rise in temperature beyond 80°C ends up resulting in a decreased KH protein yield. This may be due to extremely high temperatures and concentrations of hydrolysing agents breaking down peptide bonds which makes them lose their structure.

3.6.2. Concentration and Time. From Figure 11, it is observed that an increase in the time of hydrolysis from 1.5 hr to 3.5 hr increases the yield from 83.3% to 86.6% when the concentration of the hydrolysing agent is kept at 0.5 N. Similarly, keeping the time of extraction at 3.5 hr an increase in the concentration of the hydrolysing agent from 0.5 N to 1 N reduces the yield from 86.6% to 78.8%, showing vigorous degradation of the sheep hair sample and resulting in the loss of protein content due to further degradation of peptide bonds and hence the structure of the protein.

3.6.3. Temperature and Time. The interaction effect of temperature and time on the protein yield is shown in Figure 12. Keeping time at 1.5 hr and 3.5 hr an increase of temperature from 65°C to 80°C raises the yield from 80.1% to 82.9 to 83.3% and 86.6%, respectively. However, a further increase in temperature to 80°C drops the yields to 77.9% and 81.8%, respectively. This shows that optimality of parameters is important to get a better yield, and parameter values below or above the optimum value lead to poor extraction and denaturation of the extract, respectively.

3.7. Validation of the Model. The optimal process variables for hydrolysis of sheep hair variable numerical optimization technique based on the desirability function was carried out to determine the optimum conditions for the maximum yield of keratin protein. At the optimum hydrolysis conditions of (0.5 N, 79.7°C, and 3.4 hr) concentration, temperature, and time, respectively, protein yield was 86.6%, as shown in Figure 13. Once the optimal value of the design parameters had been selected, the final step was to predict and verify the improvement in protein yield using the optimum level of the design parameters. Therefore, to verify the validity of the study, triplicate experiments were done based on predicted values of each variable, and the protein yield was found to be 86.2%, indicating that the deviation between the predicted and actual value was calculated to be 0.56% which is less than 1%, showing that the model was satisfactory.

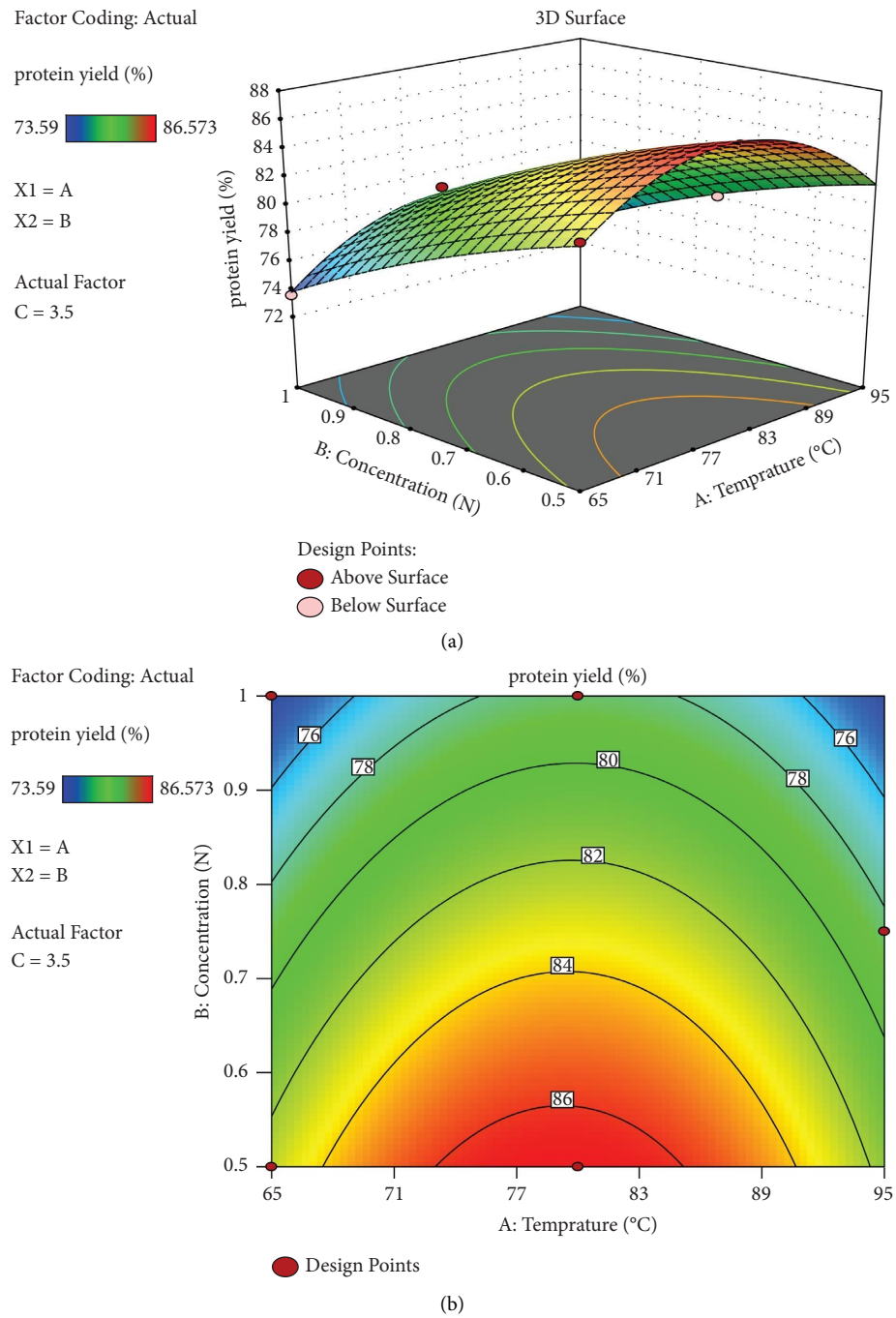


Figure 10: (a) 3D interaction effect of temperature and concentration (b) contour plot interaction effect temperature and concentration.

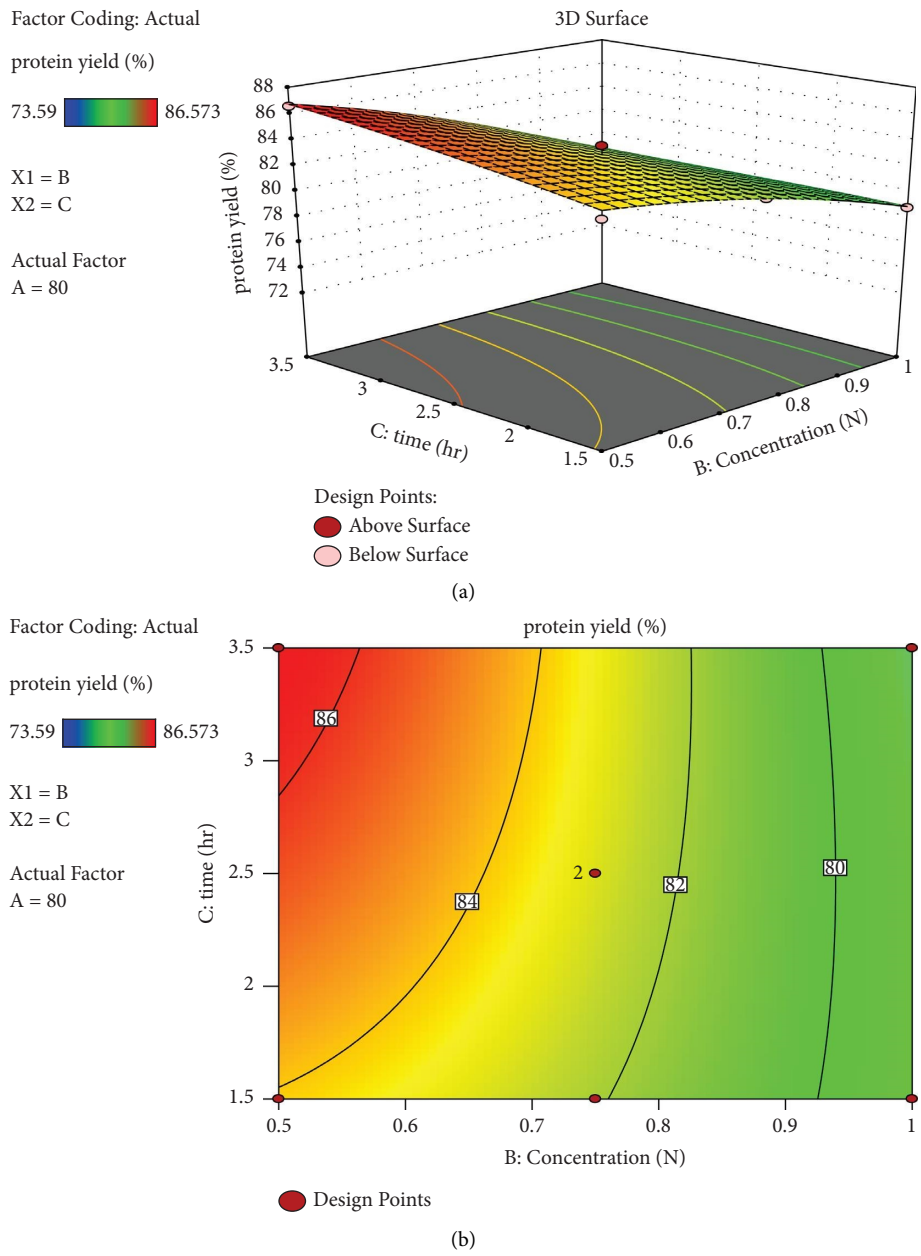


Figure 11: (a) 3D interaction effect time and concentration (b) contour plot interaction effect time and concentration.

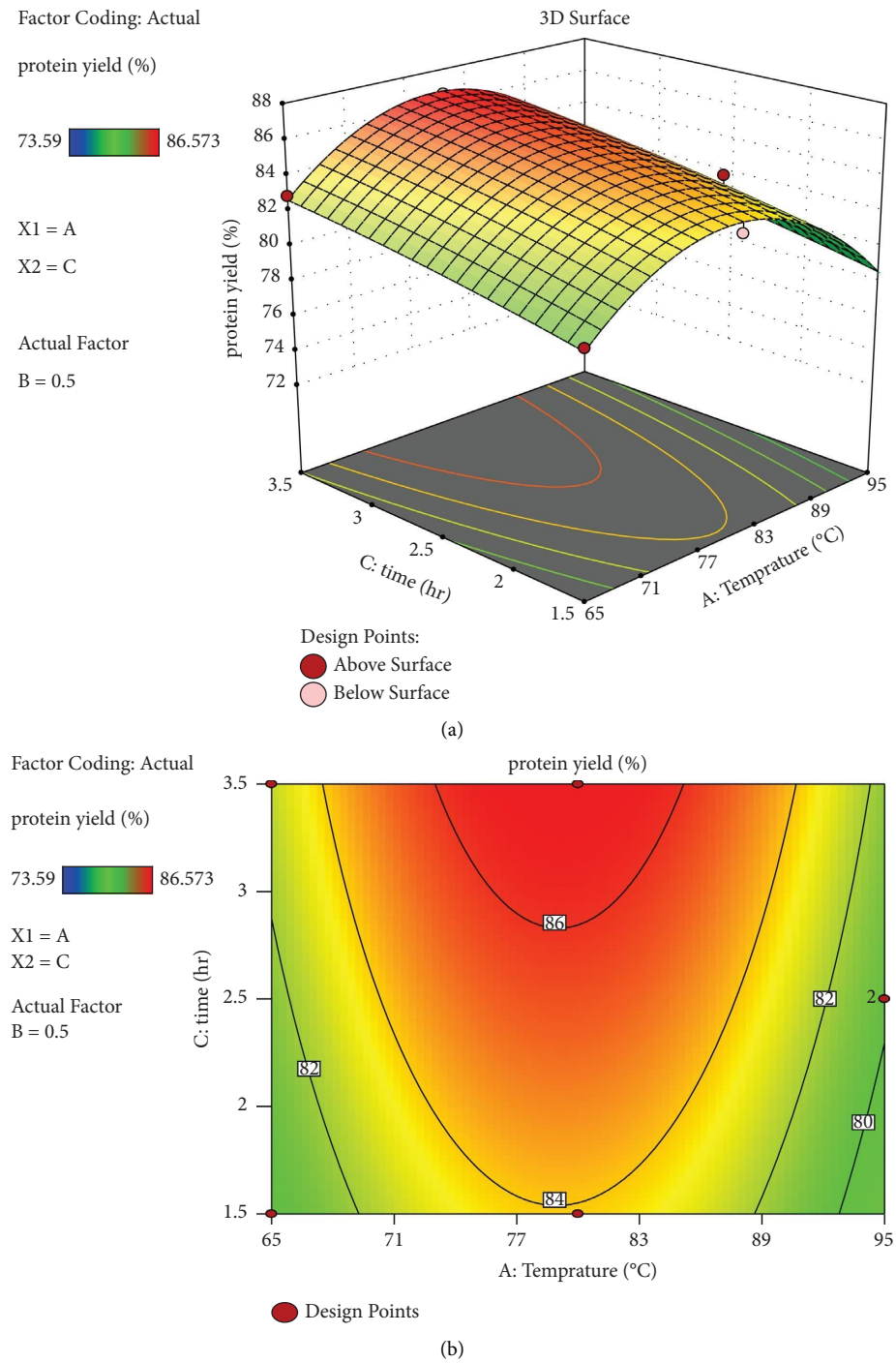


Figure 12: (a) 3D interaction effect temperature and time (b) contour plot interaction effect temperature and time.

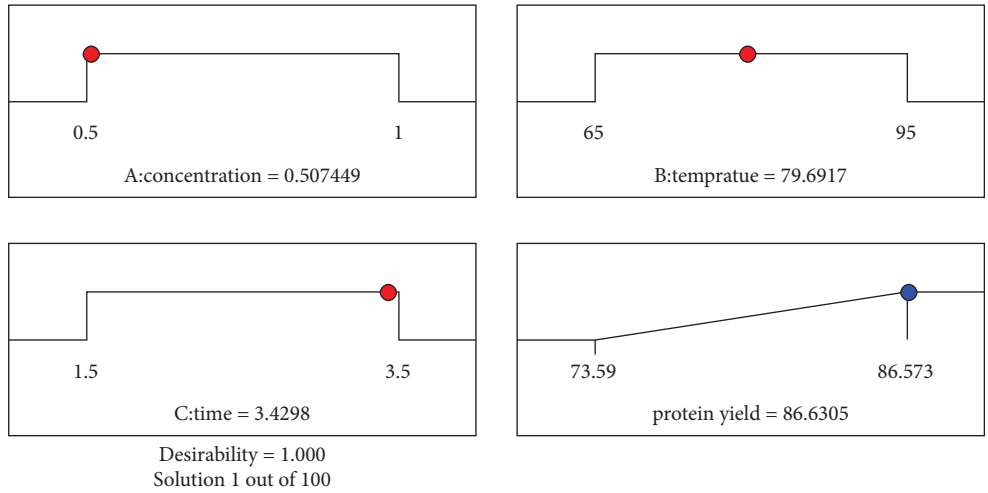


Figure 13: Ramp plot of optimization solution for the response.

4. Conclusions

Tannery hair waste was hydrolysed using alkaline hydrolysis to obtain a high yield of keratin protein considering the three main parameters. Response surface methodology with the Box-Behnken design method was used to optimize the extraction, where the maximum total nitrogen and protein content of the extract was observed to be 14.6% and 91.5%, respectively, using the Kjeldahl method, and using the biuret test method 86.57% of protein yield at optimum parameters of temperature (80°C), concentration (0.5 N), and extraction time (3.5 hr) was obtained. The scanning electron microscope (SEM) analysis showed different morphologies with the varying resolution. The FTIR analysis confirmed the presence of peptide chains and functional groups available for further reactivity, and the DLS analysis result showed allowable particle size (786.5 nm) to be used as a filler. From the SDS-PAGE analysis result, it was confirmed that the KH extract has a molecular mass in the range 1–15 kDa. Thermal stability that makes it useable for various range applications with a maximum decomposition temperature of 169.5°C was detected using DSC. It is concluded that the recycling and reuse of hair waste help not only to solve issues of solid waste handling but are also used to produce an eco-friendly retanning agent and chrome exhaust aid for leather manufacturing, thereby paving the way for cyclic economic utilization and a cleaner environment.

Data Availability

All data included inside the manuscript and there is no additional data that will be added to the manuscript.

Consent

Not applicable.

Disclosure

The research did not receive specific funding but was performed as part of the Addis Ababa Science and Technology University.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

AM, KA, and IT contributed to experimental design, experimental supervision, statistical analysis, and manuscript writing and editing. Finally, data were collected by AM.

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