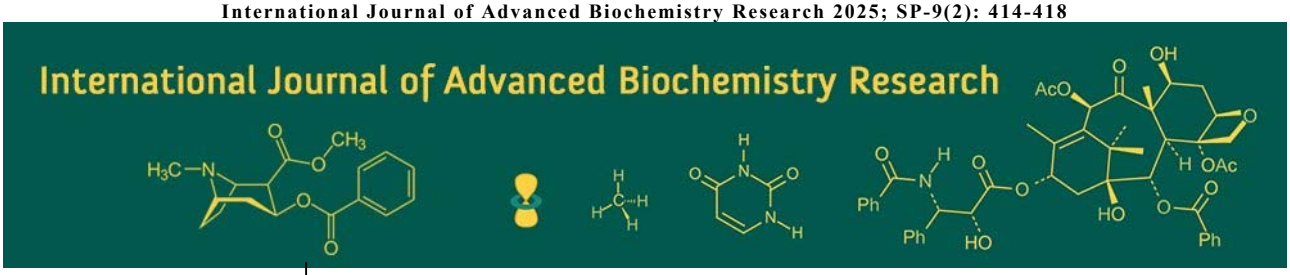

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Biodegradable Kertain bioploymers from bioconversion of poultry feathers

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Abstract
Keratin is that has been used in the production of fibrous composites, and with necessary modifications, it can be developed into gels, films, nanoparticles, and microparticles. Bioconversion of the biowaste, chicken feathers into Keratin, a multipurpose biopolymer by microbial degradation and its further conversion into biodegradable biopolymer using plasticizers such as polyethylene glycol, glycerol, and sorbitol was taken up in this study. Microbial Extraction of keratin from chicken feathers of broiler and native chicken using keratinase (from *Bacillus licheniformis*), was identified by SDS PAGE, the secondary structure was studied using Nexus FT/IR-4700 typeA spectrometer and confirmed by SEM. This research also revealed the feasibility of incorporating bioactive molecules like alkaline phosphatase into the keratin films for controlled-release applications. The study concluded that, by increasing the concentration of glycerol, the keratin sheets became more flexible and soft, also the increase of plasticizer concentration from 25 to 55 g/100 g gelatin caused an increase in flexibility and reduction of resistance and water vapor barrier.

Keywords: Chicken feather, keratin, biodegradation, biopolymer, SDS PAGE, FTIR, SEM

Introduction
The poultry industry is experiencing continuous growth, with major global producers such as the United States, Brazil, and China collectively contributing over 40 million tons of poultry meat annually (USDA, 2014) [15]. In India, poultry farming stands as one of the fastest-expanding agricultural sectors. However, poultry processing generates a substantial amount of solid waste, including feathers, viscera, bones, and dead-on-arrival birds. Among these, feather waste is particularly concerning due to its rigid structure and resistance to degradation. Feathers account for nearly 8% of a mature chicken's body weight and are composed of approximately 90% protein (Onifade *et al*., 1998) [9].

Rich in protein, feathers contain over 750 g/kg of crude protein, primarily in the form of keratin. This fibrous and insoluble protein is highly resistant to breakdown due to its extensive network of disulfide bonds, hydrogen bonds, and hydrophobic interactions. As a result, managing feather waste remains a significant environmental challenge. Although researchers have explored solutions for over two decades, no universally effective method has been widely implemented. Some potential approaches include repurposing feather waste into biofuel, biodegradable plastics, fertilizers, and electronic materials. However, converting it into feather meal remains one of the most viable options, as hydrolyzed feather meal can serve as a valuable protein source in animal feed, reducing dependence on conventional protein supplements.

Traditional methods of processing feathers, such as chemical and physical treatments, require high energy inputs and can degrade essential amino acids. In contrast, biological degradation using keratinolytic enzymes presents an efficient, eco-friendly alternative. These enzymes facilitate the breakdown of keratin, transforming feather waste into a high-protein livestock feed. This study focuses on utilizing microbial degradation techniques to extract keratin from feathers and incorporate it into biodegradable polymers with plasticizers like polyethylene glycol, glycerol, and sorbitol.

Methodology

Chicken feathers (Broiler and Desi) were collected from retail outlets in Chennai and were brought to the Department of Livestock Products Technology (Meat Science), Madras Veterinary College, Chennai. Feathers were washed thoroughly with detergent and washed several times with tap water. Washed feathers were dried in hot air oven at 80 °C for 12 hours and were used for subsequent experimentation.

Keratinase Production

Bacillus licheniformis RG1 strain was bought from MTCC (Microbial Type Culture Collection and Gene Bank). Lyophilized bacteria were revived in 50 ml nutrient broth. Later subculturing was done by inoculating 1% v/v bacterial culture in 50 ml nutrient broth in Ehrlenmeyer flask and incubated at 37 °C for 24 h at 200 rpm speed of agitation and was used as seed culture. This seed culture was used as inoculum for production medium. (Tiwary and Gupta, 2012) [14]. After 72 hours of keratinase production, the culture broth was collected in a 500ml beaker and kept undisturbed for one day at room temperature (25 °C). Most of the bacteria were settled along with feather meal. The supernatant was syphoned off and micro filtered through 0.2 µ filters using a vacuum pump. The micro filtered supernatant was then concentrated (using ammonium sulphate) and used as enzyme. All the solutions required for microbial degradation were prepared just before the treatment by using cold distilled water and kept in chiller at 4 °C to maintain the chillness prior to use.

Extraction of keratin from feathers by microbial Keratinase

Extraction of keratin was done according to the method described by Tiwary and Gupta (2012) [14] with slight modifications. Each trial was done with 2 g of pre-weighted feather in a glass conical flask at 50 °C in the shaker incubator. One hundred ml of already prepared keratinase enzyme and equal volume of phosphate buffer (pH 8) was added to the precooked feather and mixed thoroughly. It was kept at 50 °C and 200 rpm till complete degradation of feathers. It was then filtered through 2mm sieve and the filtrate was collected and centrifuged at 10000 rpm for 20 minutes. The keratin was precipitated using 10% TCA at 4 °C and was kept undisturbed for 12 hrs. It was then centrifuged at 10000 rpm for 20 mins. Later the sediment (keratin) was concentrated using dialysis. Dialysis was done in dialysis membrane made of regenerated seamless cellulose tubing wherein the membrane was partially permeable, having molecular weight cut off of 12 kDa. The dialysed hydrolyzates were filled to three-fourth in 10 ml vials and sealed with rubber stopper and kept in deep freezer at-22 °C overnight. The pre-freezed vials were lyophilized in the crystal lyophilizer at-54 °C for 24 to 32 hours. The lyophilized keratin was stored at 4 °C.

Physico-Chemical Characteristics

The keratin yield (%) was calculated after lyophilization. The crude keratin was calculated using the following formula:

$$\text{Keratin yield (\%)} = \frac{\text{Weight of lyophilized dry crude keratin}}{\text{Weight of feathers}} \text{ X } 100$$

The pH of crude keratin from the degraded feather was measured at the end of lyophilization according to the procedure USP monograph 39-NF 34 by using a digital pH meter (Digisun Electronic System, Model: 2001). Colour of keratin was measured using Hunter colour lab Mini scan XE plus Spectro-colorimeter (Model No. 45/O-L, Reston Virginia, USA) with geometry of diffuse/80 (sphere-8mm view) and an illuminant of D65/10 degree (USP monograph 39-NF 34). The feather and their hydrolyzate were analysed for proximate composition such as protein and fat by following the standard procedure of AOAC (1995). Characterization of the obtained keratin was done by SDS-PAGE in Mini Vertical Gel Electrophoresis unit (SDS-PAGE apparatus, Model Regular-Mini Vertical, ORANGETM, Chennai). Further studying of the secondary structure of keratin was done by FTIR spectroscopy.

FT-IR spectra of keratin were recorded from 400 to 4000 cm-1 using a Nexus FT/IR-4700 type A spectrometer. For solid-state measurement, a pellet was prepared by mixing lyophilized keratin with potassium bromide. Samples were prepared for FT-IR according to the method described by Khan *et al*. (2013) [6]. The lyophilised sample was plated with Eiko IB-5 ion coater. The specimens were then observed with XL30-ESEM environment scanning electron microscopy under various resolutions.

Keratin Biopolymer

10 ml of the aqueous dispersion of the reduced keratin (7 g keratin/100 ml dispersion) was taken in a 50ml beaker and was kept in magnetic stirrer. It was then mixed with glycerol in the following concentrations: 0.03, 0.05, 0.07 and 0.09 g/g of keratin. Glycerol was added to the dispersion drop wise while the stirring is in process. Silicone oil was applied using cotton on to a polystyrene petri dish (8 cm diameter), to which these film forming dispersions were poured and dried in a ventilated oven at 30 °C for 24 h (Martelli *et al*. 2006) [8]. In the present study, the plasticizer used was glycerol (98 percent pure). The keratin biopolymers were prepared by adding various concentration of glycerol (0.03, 0.05, 0.07 and 0.09 g/g of keratin) by constant stirring. We concluded that, by increasing the concentration of glycerol, the keratin sheets became more flexible and soft.

Statistical Analysis

The data was subjected to statistical analysis in SPSS (version 20.0) software as per the standard procedure outlined by Snedecor and Cochran (1994) [13].

Results and Discussion

The mean ± SE value of yield percent, pH, Lightness, Yellowness, Redness, Hue, Chroma, Protein and Fat of keratin extracted from broiler and native chicken feathers are given in Table 1.

The test of significance revealed no significant difference (p>0.05) in the yield percent, pH, Redness, Yellowness, Hue and Chroma values of keratin extracted from both broiler and native chicken feathers. The test of significance revealed highly significant difference (P<0.01) in lightness value of keratin extracted by microbial degradation from broiler and native chicken feathers.

The present study revealed that highest yield in percentage of keratin was observed when an inoculum size was 4 percent and at temperature of 37 °C at pH 8 using keratinase obtained from *Bacillus licheniformis*. Werlang and Brandelli (2005) [16] degraded feather using *Bacillus sp*. Strain and obtained the maximum protein concentration of 6.8 g/L, at

72 h of activity. Maximum growth and feather-degrading activity were observed at 30-37 °C. The pH of keratin from these feathers did not differ significantly and observed results were agreeing with the findings of keratin isolated from feather which was 7.5-8.5

(Cai *et al*. 2008) [2]. Werlang and Brandelli (2005) [16] correlated pH with keratin yield and found that the optimum pH for the keratinolytic enzyme to act on feathers were 8.0-11.0.

Lighter colour of broiler keratin gives an advantage as it does not affect the colour of the finished product. There is scanty literature on the studies of colour of keratin from chicken feathers.

However, in the present study, the keratin extracted from broiler feathers was white/whitish in colour indicating that the removal of fat and other pigments during the process of extraction was not efficient.

The mean ± SE values of protein and fat content of keratin extracted by microbial degradation from broiler and native chicken feathers are presented in Table 1. The test of significance revealed that no significant difference (p>0.05) was observed in protein and fat content of keratin extracted by microbial degradation from broiler and native chicken feathers.

The mean ± SE value of Bradford assay of keratin extracted by microbial degradation from broiler and native chicken is as shown in Table 2 and Table 3. The test of significance revealed highly significant difference (p>0.05) in Bradford assay values (protein concentrations) of keratin extracted by microbial degradation from broiler and native chicken feathers. (Yi-Chun *et al*. (2004) [18] determined the keratin protein concentration in a tape-stripped skin sample through bradford assay and found that the protein concentration ranges from 154±75.3 µg/cm^2 to 52.7±17.3 µg/cm^{2}which was near to the present studies' protein concentration values.

The SDS-PAGE patterns of keratin extracted from chicken feathers by microbial degradation are shown in the plate 1. The SDS-PAGE showed bands in lane 1 representing the protein marker were recorded in twelve bands with the molecular weight of 196, 102, 75, 57, 41, 28, 20, 15 and 6 kDa in sequential order whereas keratin extracted from chicken feathers by microbial degradation the in lane 2 depicted a band with molecular weight of 20kDa and also a band with the molecular weight ranged between 41 kDa to 57 kDa. In the present study, the keratin extracted from chicken feathers by microbial degradation had 2 bands, α-keratin having a molecular weight of approximately 50 kDa and β-keratin having the molecular weight of 20 kDa. There is scanty literature on the studies of molecular weight of keratin from chicken feathers. However, Rouse and Dyke (2010) could observe that the keratin obtained from chicken feathers were having 2 bands with molecular weight of 20 and 50 kDa and this finding was very much similar to the present study.

FTIR spectra of keratin exhibited characteristic peaks of-CONH vibration of amide group, C=O stretching vibration, S=O stretching vibrations,-C-O-S,-COO; C-C and R-SO2-R groups as shown in Table 4 and Figure 1.The transmittance peaks for the amide I (1665 cm-1) and amide II (1533 cm-1) indicates the presence of an α-helix structure in the sample, moreover the amide I (1638 cm-1) and amide II (1515 cm-1) peaks indicate the presence of a β-sheet type. Peaks that appear between 1200 and 1000 cm-1 (1078 cm-1) are attributed to the S=O vibration The peaks appeared in the range of 480-560 cm-1 as shown in Figure 1. represented that disulphide bonds existed in the sample.

The characteristic peaks of-CONH was observed at 1665 cm-1 for standard C-S.

Characteristic peaks of C-O-S, SO2 and SO were observed at 831 cm-1 for extracted samples of keratin and characteristic peaks of N-H, CN and C=O was observed at 1331 cm-1 for extracted keratin samples. Similar to the present study conducted, Gupta *et al*. (2011) [4] confirmed that the product obtained is keratin through FTIR. The presence of carboxyl group and amino groups, the two groups that will only be present in amino acids were confirmed by analyzing the wavelengths representing C=N bond, N-H bond, and C=O bonds. Lo *et al*. (2012) [7] also detected similar functional groups of keratin obtained in our study by FT-IR.

Scanning electron microscope picture revealed spherical, tightly packed nanoparticles and random arranged porous microstructures which were characteristic of keratin protein (Plate 2). Rayudu (2015) [11] observed that degradation of feathers could be seen through scanning electron microscope after 72 h. Feather barb and opening of barbules and partial degraded feather barbules could be observed after 48 h of incubation. Similar results were observed by Jeong *et al*. (2010b) [5] where most of the feathers were degraded after 3 days except the feather shaft, which was completely degraded after 6 days of incubation. In the present study, keratin obtained by degradation of feathers were observed under Scanning Electron Microscope and observed round tiny nano particles adhered together which indicated the fine degradation of feathers.

Orliac *et al*. (2003) [10] concluded that, out of all the plasticizers used (ethylene glycol (eg), propylene glycol (pg), polyethylene glycols (PEGs), polypropylene glycols (PPGs)), the plasticizers which adapted best to protein (Sunflower Protein Isolate) seemed to be Triethylene glycol and glycerol while preparing biopolymers. Fujii *et al*. (2004) [3] demonstrated that hair keratins were useful for preparing protein films and described a rapid casting method. This research also revealed the feasibility of incorporating such bioactive molecules as alkaline phosphatase into the keratin films for controlled-release applications.

The films, however, had poor strength and flexibility.

Table 1: Mean ± SE of physico chemical and proximate composition (dry weight basis) of keratin extracted by microbial degradation of broiler and native chicken feathers

Parameters	Chicken feathers		t-value
	Broiler	Desi	
Yield (%)	11.64±0.28	11.06±0.40	**1.20^{NS}**
pH	8.3±0.05	8.3±0.06	0.07 ^{NS}
Lightness	92.60 ±0.98	50.97±1.08	28.58**
Redness	4.10±0.21	3.84±0.08	1.17 ^{NS}
Yellowness	19.25±0.04	16.23±0.98	3.06 ^{NS}
Hue	77.97±0.62	76.64±1.04	1.10 ^{NS}
Chroma	16.67±2.05	15.77±1.03	0.39 ^{NS}
Protein	79.75±1.65	80.50 ±1.75	0.22 ^{NS}
Fat%	17.2±0.05	15.4±0.06	0.08 ^{NS}

NS-Not Significant *-Significant (p<0.05) difference **-Highly significant (p<0.01) difference

Means bearing different superscripts in the same row differ significantly

Table 2: Preparation of standard keratin (chicken feather) for Bradford Assay

Final Concentration (mg/ml)	Volume of 10 mg/ml Standard added (µl)	Volume of Dilution Buffer (µl)	Reading at A_{595}
0 (Blank)	0	1000	0.02
Protein sample	100	900	0.335
1	100	900	0.845
0.5	50	950	0.67
0.25	25	975	0.605
0.125	12.5	987.5	0.475
0.0625	6.25	993.75	0.305
0.03125	3.125	996.875	0.225

Table 3: Mean ± SE values of Bradford Assay (mg/g) of keratin extracted by microbial degradation of broiler and native chicken feathers

Parameters	Chicken feathers		t-value
	Broiler	Desi	
Keratin concentration (µg/ml)	462.81±0.56	465.29±0.27	3.98**

NS-Not Significant *-Significant (p<0.05) difference **-Highly significant (p<0.01) difference
Means bearing different superscripts in the same row differ significantly

Table 4: Characteristic peaks of Standard keratin and keratin extracted by microbial degradation of chicken feathers at mid FT-IR region (400-4000 cm^{-1})

Characteristics	Wave numbers (cm^{-1})	
	Standard	Keratin sample
Amide A-NH stretching, OH bond	3432	3429
CH_3 and CH_4 stretching	2918	2922
Amide I-(C=O stretching, C-N , N-H linkage)	1700-1600	1665
Amide II (C-N , N-H linkage)	1560-1500	1533
Amide III(C=O, O=C=N, C-N , N-H linkage)	1400-1300	1331
Cysteine, R-SO_2-R; R-SO-R, S=O	1044	1078
Amide IV (C-S)	675	681

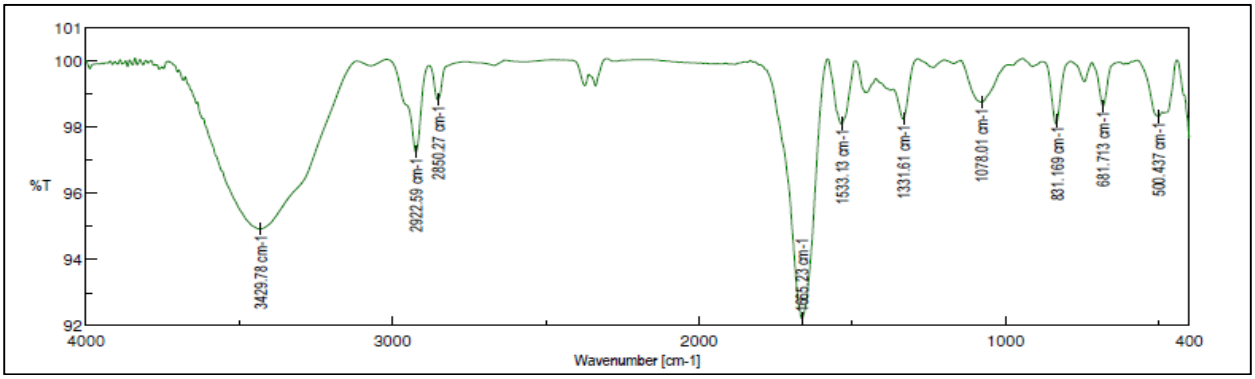


Fig 1: FT-IR spectrum of keratin extracted by microbial degradation of feathers (broiler and native)

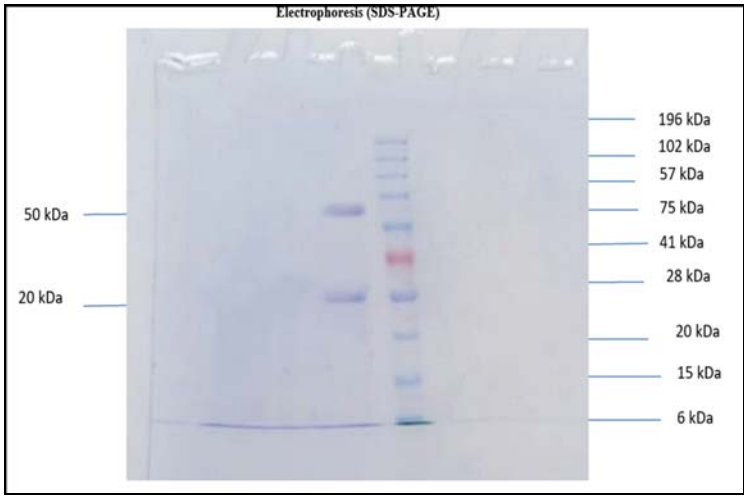
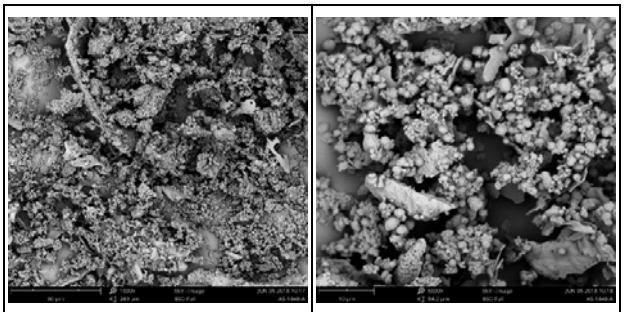


Plate 1: Molecular pattern of keratin obtained by chicken feathers on Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)



Under 1000x magnification	Under 5000x magnification

Plate 2: Scanning Electron Microscope pictures at various resolutions

Conclusion

The growing need for sustainable alternatives in packaging has led to increased interest in biodegradable natural polymers. As food industry demands, industrial production methods, and consumer preferences evolve, the packaging sector must shift towards eco-friendly solutions. This study demonstrates that chicken feathers can be effectively processed to extract keratin, which can be repurposed into valuable materials. Efficient collection and utilization of feather waste will help reduce environmental pollution by preventing its accumulation in landfills and water bodies. The resulting keratin-based biopolymers have versatile applications, including food packaging, wound healing, controlled drug delivery systems, and cosmetic formulations.

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