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Microbial degradation of chicken feathers to convert waste into value added biopolymer

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Abstract
A study on the bioconversion of chicken feathers (Broiler and Desi) by microbial degradation to extract keratin and in turn produce biopolymer was carried out in the Department of Livestock Products Technology (Meat Science), Madras Veterinary College, Chennai-7. Seven trials were carried out each with the broiler and desi feathers. The extracted keratin was lyophilized and evaluated for pH, yield, protein, fat and instrumental color analysis. The molecular characteristics were also studied by using FT-IR. The study revealed that there was no significant difference (P>0.05) in the yield, pH, protein, fat, redness, yellowness, hue and chroma values of keratin extracted from both the chicken feathers. There was a high significant difference (P>0.05) between lightness of keratin extracted from broiler and desi chicken feathers. FT-IR spectroscopy analysis of keratin indicated the presence of an α-helix structure, a β-sheet type and the disulphide bonds existing in the sample which characterized that the extracted content was keratin. Keratin biopolymer was prepared from the extracted keratin by a casting technique.

Keywords: Keratin extraction, feather, microbial degradation, FTIR analysis

Introduction

India's broiler production for calendar year (CY) 2018 reached 4.6 million tons on increasing demand from the growing middle class (USDA Foreign Agricultural Service)). The continuous growth of poultry industry results in an increased amount of waste generated from production and processing units. The poultry meat processing results in massive quantities of solid waste as feathers, viscera, bones and dead on arrival (Brandelli *et al.*, 2015) [8]. These large amount of feathers that is obtained as a by-product from the poultry processing plant is of relatively low value, which most of the times is being discarded as waste resulting in environmental pollution and protein wastage. The use of bio-enzymes for bio-conversion of such byproducts into materials with increased value is an interesting strategy.

Traditional methods such as steam, pressure and strong alkali or acid usually used in processing feathers required significant energy and resulted in destruction of some essential amino acids (Papadopoulus, 1989). The most important factor affecting the quality of hydrolyzed poultry feathers is the extent of hydrolyzation. If less than 75 % of the crude protein content is digestible by the pepsin digestibility method, then hydrolyzation was incomplete and protein quality is reduced. Physical or chemical treatments of feather processes require significant energy and also destroy certain amino acids. Biological degradation of feather waste is more efficient than both the physical and chemical degradation methods because it yields more useful and chemical free by-product. (Williams *et al.*, 1990) [4].

Feathers are an abundant waste of poultry industry since they account for approximately 8% of the adult chicken weight and are constituted by about 90% protein (Onifade et al., 1998) [5]. Feathers are bio-resources with high protein content of more than 750 g per kg crude protein. These feathers contain good amount of keratin, mainly as β-keratin. Keratin shows an elevated level of amino acids such as glycine, alanine, serine, cysteine and valine but low amount of lysine, methionine and tryptophan. Some microbial enzymes can hydrolyze insoluble feather keratins, allowing their conversion into feedstuffs, fertilizers, and films (Gupta and Ramnani, 2006; Onifade *et al.*, 1998) [5, 6]. In addition, applications of these enzymes for pharmaceutical and cosmetic purposes have been also described (Brandelli, 2008) [8]. Keratins are insoluble fibrous proteins, highly cross-linked with disulfide bridges, hydrogen bonds, and hydrophobic interactions.

The tightly packed super coiled polypeptide chains result in high mechanical stability rendering it resistant to proteolysis by common proteases such as trypsin, pepsin, and papain. These keratinolytic enzymes may have important applications in biotechnological and industrial processes involving keratin-containing wastes from the poultry and leather industries through the development of non-polluting processes and dehairing of skin and hides. Keratin extracted from feathers may also be used to develop biodegradable polymers using plasticizers such as polyethylene glycol, glycerol and sorbitol. Biodegradable polymers find applications in emerging technologies including tissue engineering and regenerative medicine, gene therapy, novel drug delivery systems, implantable devices and nanotechnology.

Materials and Methods

Bacterial Strain: The bacterium, *Bacillus licheniformis* was acquired from MTCC (Microbial Type Culture Collection and Gene Bank). Lyophilized bacteria were revived in 50 ml nutrient broth. Stock culture of the strain was stored at -22ºC in 50% glycerol.

Inoculum preparation and keratinase production: For seed culture preparation, 1% v/v bacterial culture was grown at 37ºC, 200 rpm for 24 hrs in 50 ml nutrient broth in Ehrlenmeyer flask. Again this seed culture was grown in nutrient broth for 24h at 37 ºC and was used as inoculum for production medium. For keratinase production, the production medium of 150 ml with pH 7 was dispensed in 250 ml Ehrlenmeyer flasks and sterilized by autoclaving at 121ºC, 15psi for 20 min. The production medium (150ml) contains: 0.5% feather, 0.4% soy flour, 3.2% glucose, 0.3% KH_2PO_4 and 0.6% K_2HPO_4(Tiwary and Gupta, 2012). Feather was used as the only source of nitrogen and carbon. Each flask was then inoculated with 2% v/v of 24h old seed culture prepared in nutrient broth. The samples were then incubated at 37ºC, 200rpm for 72h.

After 72h of keratinase production, the culture broth was collected in a 500ml beaker and kept undisturbed for one day at room temperature (25ºC). The supernatant was syphoned off and micro filtered through 0.2µ filters using a vacuum pump. The micro filtered supernatant was then treated with 1% lysozyme for 1 hour and centrifuged at 9500 rpm at 4°C. The supernatant was then used as keratinase enzyme.

Keratinase Assay: The protease activity was estimated according to Secades and Guijarro (1999) [2]. The keratinolytic activity of keratinase was determined using keratinase assay with keratin as a main substrate. The reaction mixture containing 4ml of Glycine-NaOH buffer (pH 10) was incubated along with 20 mg of keratin and 1 ml of keratinase enzyme for 60 min at 60ºC. The reaction was stopped by adding 4 ml of 5% (w/v) TCA and then incubated at 25 ºC for 60 min. The insoluble residue was filtered through a filter paper and the reaction mixture was centrifuged at 8000 rpm for 10 mins. The supernatant was then used to read the absorbance at 280 nm. Control was prepared in the same way except that 1% (w/v) TCA was added along with 3ml of Glycine-NaOH buffer instead of 4ml buffer. One unit (U/ml) of keratinolytic activity was defined as an increase of 0.01 in the absorbance at A280.

Feather Degradation

2gm of feather was soaked in 200ml water for 2 hrs. It was then boiled for 20 minutes and then cooled to room temperature. 100 ml of already prepared keratinase enzyme along with equal volume of pH 8 phosphate buffer was added to this and mixed thoroughly. It was kept at 50ºC and 200rpm till complete degradation of feathers. It was then filtered through 2mm sieve and the filtrate was collected and centrifuged at 10,000 rpm for 20 minutes. The keratin was precipitated using 10% TCA at 4°C and was kept undisturbed for 12hrs. It was then centrifuged at 10,000rpm for 20 minutes. Later the keratin (sediment) was concentrated using dialysis.

Dialysis membrane and its closure clips (HIMEDIA® Pvt. Ltd), with a capacity of approximately 3.63 ml/cm was used. According to the final volume obtained, after obtaining the protein precipitates, the dialysis membrane length was pre assumed and dialysis membrane was cut and pre wetted prior to use. 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.

Keratin Yield (%)

The keratin yield (%) was calculated after lyophilization. The crude keratin was calculated using 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 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). The feather and their hydrolyzate were analysed for protein and fat by following the standard procedure of AOAC (1995). Fat and protein estimation were estimated using SOCS plus (Model SCS 4, Pelican Equipment Pvt. Ltd., Chennai) and KEL plus (Model Classic DX, Pelican Equipment Pvt. Ltd., Chennai) equipment, respectively. 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 deg (*USP monograph 39–NF 34*).

Colorimetry measures colour quantitatively and can define them within well-established numerical values. They were expressed using the standard Huntercolour L* a* b* system. L*, a*, b* values (non-dimensional units) refer to the axes of the system: lightness axis, (white – black, L*); and two axes representing both hue and Chroma, one red - green (a*) and other blue – yellow (b*). This system provides an unambiguous description of colour and has the advantage that colour differences between samples can be determined using simple computer programs.

The instrument was calibrated with black and white tile (L* = 94, a* = 1.10 and b* = 0.6) every time before the colour measurement was taken. The colour was expressed as L (brightness), a*(redness) and b* (yellowness). The hue (relative position of colour between redness and yellowness) and chroma (colour intensity) was calculated as follows.

$$\text{Hue} = \tan^{-1}(b^*/a^*)$$
$$\text{Chroma} = \sqrt{(a^*)^2 + (b^*)^2}$$

Average value for each colour parameter was determined by taking observation from lyophilized chondroitin sulphate of each trial.

FT-IR spectroscopy
FTIR 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. All spectra were measured at a resolution of 4 cm^{-1}.

Preparation of feather keratin films
The aqueous dispersion of the reduced keratin was mixed withglycerolin the following concentrations: 0.03, 0.05, 0.07 and 0.09 g/g of keratin. The mixture was stirred in magnetic stirrer for 1 h at room temperature. These film-forming dispersions were casted into polystyrene petri dishes (10cm diameter) and dried in a ventilated oven at 40 °C for 24 h. The films were then removed from the casting surface (petri dish) and stored. The thickness of the films was controlled byvarying the volume of the keratin dispersion poured into thepetri dish.

Results and Discussion
Keratin Yield (%): The mean ± SE value of yield of keratin % extracted from broiler and desi chicken feathers were 11.64 ± 0.28 and 11.06 ± 0.40 per cent, respectively.

pH: The mean ± SE value of pH of keratin extracted by microbial degradation from broiler and desi chicken feathers were 8.3 ± 0.05 and 8.3 ± 0.06, respectively.

Instrumental Colour Analysis: The mean ± SE values of Lightness (L*) of keratin extracted by microbial degradation from broiler and desi chicken feathers were 92.60 ± 0.98 and 50.97 ± 1.08, respectively. The test of significance revealed highly significant difference ($P>0.05$) in lightness value of keratin extracted by microbial degradation from desi chicken feathers and broiler chicken feathers.
The mean ± SE values of Redness (a*) of keratin extracted by microbial degradation from broiler and desi chicken feathers were 4.1 ± 0.21 and 3.84 ± 0.08, respectively. The mean ± SE values of Yellowness (b*) of keratin extracted by microbial degradation of broiler and desi chicken feathers were desi chicken feathers were 19.25 ± 0.04 and 16.23 ± 0.98, respectively. The test of significance revealed no significant difference ($P>0.05$) in redness and yellowness values of keratin extracted by microbial degradation from desi chicken feathers and broiler chicken feathers.
The mean ± SE values of Hue of keratin extracted by microbial degradation from broiler and desi chicken feathers were 77.97 ± 0.62 and 76.64 ± 1.04, respectively. The mean ± SE values of Hue of keratin extracted by microbial degradation from desi chicken feathers were. The mean ± SE values of Chroma of keratin extracted by microbial degradation from broiler and desi chicken feathers were 16.67 ± 2.05 and 15.77 ± 1.03, respectively. The test of significance revealed no significant difference ($P>0.05$) in hue and chroma values of keratin extracted by microbial degradation from desi chicken feathers and broiler chicken feathers.

Protein (%): The mean ± SE value of protein content of keratin extracted by microbial degradation from broiler and desi chicken feathers were 79.75 ± 1.65 and 80.50 ± 1.75 per cent.

Fat (%): The fat content of keratin extracted by microbial degradation from broiler and desi chicken feathers were 17 ± 0.05 and 15 ± 0.06 per cent.

FTIR: The keratin extracted by microbial degradation from chicken feather was identified by FT-IR spectroscopy technique using potassium bromide pellet by comparing with standard keratin at 400-4000 cm^{-1}.FTIR spectra of degraded feather displayed that transmittance peaks nearby 500, 681, 831, 1078, 1331, 1533, 1665, 2850, 2922 and 3429 cm^{-1}. The amide A band at 3429 cm^{-1} is connected with the stretching vibration of the N-H bond. The peak located in the range of 2700-3100 cm^{-1} indicates the presence of CH groups, and the broad peak around 3400 cm^{-1} is usually caused by the vibration of hydrogen bonded –OH groups. The transmittance peaks for the amide I (1665 cm^{-1}) and amide II (1533 cm^{-1}) suggest the presence of an α-helix structure in the sample, moreover the amide I (1638 cm^{-1} and amide II (1515 cm^{-1}) 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 Fig. represented disulphide bonds existing in the sample. FTIR spectra of keratin exhibited the characteristic peaks of **–**CONH vibration of amide group coupling of C-O stretching vibration, S=O stretching vibrations, **-**C**-**O**-**S, **-** COO; C**-**C, C**-**O**-**S and R**-**SO2**-**R; R**-**SO2**-**R. The characteristic peaks of –CONH was observed at 1665 cm^{-1} for standard C-S as shown in fig 1. Characteristic peak of C-O-S was observed at 831 cm^{-1} for extracted samples of keratin and characteristic peak of S=O was observed at 1331 cm^{-1} for extracted keratin samples.

Keratin Biopolymer: By increasing the concentration of plasticizer, the flexibility of biopolymer increased. Similar conclusion was provided by Thomazine *et al.* (2005) [7]. They concluded that the increase of plasticizer concentration from 25 to 55g/100g gelatin caused an increase of flexibility and reduction of resistance and water vapor barrier.

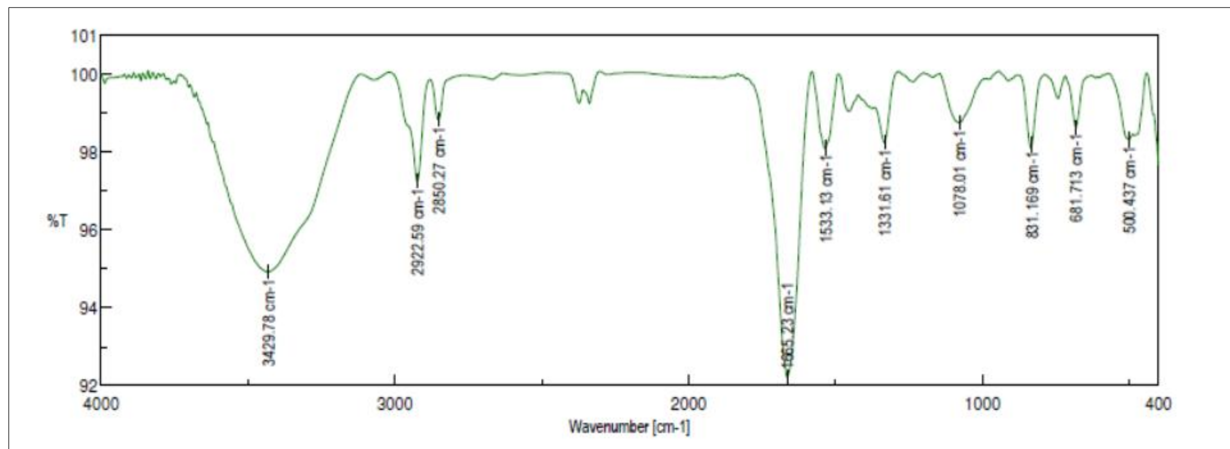

Fig 1

Table 1

Parameters	Broiler	Desi	t-test
Keratin Yield (%)	11.64 ± 0.28	11.06 ± 0.4	1.20^{NS}
pH	8.3 ± 0.05	8.3 ± 0.06	0.07^{NS}
Protein	79.75 ± 1.65	80.5 ± 1.75	0.22^{NS}
Fat	17 ± 0.05	15 ± 0.06	0.08^{NS}
Lightness	92.6 ± 0.98	50.97 ± 1.08	28.58^{**}
a*	4.1 ± 0.21	3.84 ± 0.08	1.17^{NS}
b*	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}

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

Discussion

The keratin yield obtained was near to 11% for both broiler and desi feathers, whereas it was less compared to the procedure followed by Tiwary and Gupta, 2012. The lightness of keratin extracted from broiler feathers were more when compared to keratin extracted from desi feathers. 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. In the present study, the keratin extracted from broiler feathers is white/whitish in colour indicating that efficient removal of fat and other pigments during the process of extraction and the keratin extracted from broiler feathers is white/whitish in colour indicating that the removal of pigments was not possible during the process of extraction.

Conclusion

The protein based biopolymers have emerged as potential substitutes for many biomedical and biotechnological applications due to their ability to function as a synthetic extra cellular matrix that facilitates cell to cell and cell matrix interactions. Also, keratin based biopolymers will bring immense changes due to their intrinsic biocompatibility, biodegradability, mechanical durability and natural abundance. Though, keratin biomaterials have been in the collective conscience of researchers for many decades, yet there are no keratin biomaterials currently in clinical use. This study will make these keratin biopolymers break their way into clinical trials.

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