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Bioconversion of poultry feather into feather meal using proteolytic *Bacillus Species*: A comparative study

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Abstract
The thermo-energetic cost of conventional feather processing has paved way for microbial enzyme technology, where bioconversion of poultry feathers into a nutritionally enhanced feather meal can be achieved. A beneficiary attempt has been made to isolate proteolytic bacteria from tannery lime effluent, as it is a keratin-rich man-made source. Isolates – T1, T2 and T3 that showed vibrant clear zone of hydrolysis on skim milk agar plates, were identified as *Bacillus subtilis, Bacillus flexus and Bacillus endophyticus* respectively, through MALDI-TOF. Caseinolytic activity of the isolates on milk agar plates proved them as extracellular protease producers. Maximum solubilization of feathers was achieved with *Bacillus flexus*. Biodegradation of chicken feather was found to be 59%, 68% and 47% by *Bacillus subtilis, Bacillus flexus and Bacillus endophyticus* respectively. The proteolytic enzymes from the isolates were partially purified and preserved for further characterization.

Keywords: *Bacillus*, Biodegradation, Poultry feathers, Proteolytic enzymes, Solubilization

1. Introduction
Poultry feathers, made up of 91% of hard keratin in them, are the major waste generated in the poultry farms, due to the processing of chickens for meat. Keratin is a complex, rigid protein made up of α helix and β sheets, which are tightly bonded with disulfide bonds. This rigid bonding of the keratin structure plays a crucial role in the birds' locomotion and protection. Due to the complex protein structure, keratin is generally resistant to proteolysis. Although utilization methods such as incineration, controlled land filling, rendering and anaerobic digestion/composting are practiced in the poultry farms, disease ailments among workers and environmental contamination of the nearby areas still exist. Emission of toxic gases like ammonia and hydrogen sulfide, with obnoxious odors contributes to severe illness and infections.

Conventional processing of feathers, results in the destruction of essential amino acids, as the digestion of feathers is performed at high temperatures and under high pressure. Chemical hydrolysis of chicken feathers is done using calcium hydroxide. At high temperatures (150 °C), 80% of feather keratin is solubilized within 25 min. However a relatively longer reaction time (300 min) is needed at moderate temperatures (100 °C). After 3 h of hydrolysis at 150 °C, 95% of feather keratin is digested [4]. As an eco-friendly alternative to hydrothermal and chemical hydrolysis, microbial enzyme-mediated solubilization of feathers is now a subject of great interest. Bioconversion of feathers into feather meal will retain intact essential amino acids of good nutritional value. Microbial biomass will contribute to the enhancement of the protein content of the feather meal and improve digestibility. Protein hydrolysates obtained after microbial keratinolysis will account for higher nutritional value of the feather meal, which can act as a source of protein in poultry/aqua feeds.

In this present paper, an attempt has been made to isolate protease producing microorganisms from tannery lime effluent and to evaluate their biodegradation activity on chicken feathers. With this aim in mind, the main objectives of this study are:

i) to isolate proteolytic bacteria from tannery lime effluent and its identification using MALDI-TOF;
ii) inoculum preparation
iii) to examine the enzymatic degradation of chicken feathers by proteolytic bacteria and to calculate the degree of degradation (DD) of feathers and
iv) Partial purification of the protease enzymes of all the proteolytic strains.

2. Materials and Methods
2.1 Sample Collection
For the isolation and identification of proteolytic bacteria, tannery lime effluent sample was collected in sterile sampling bottles from E.K.M. Leather Processing Company, Khaleel Tanning Company, in Erode District of Tamil Nadu, India. The samples were transferred immediately to the laboratory for further analysis.

2.2 Isolation of Microorganisms from Lime Effluent
Serial Dilution was performed to enumerate the total number of viable cells present in the lime effluent [3]. About 1ml of the sample was added to 9ml of sterile distilled water and this suspension was serially diluted. About 0.1 ml from each dilution was spread plated onto sterile nutrient agar plates and incubated at 37°C for 24 hours. Total number of viable cells is counted using the following formula –

$$\text{Total no. of viable cells} = \text{Average no. of colonies} \times \frac{\text{Dilution factor}}{\text{Inoculum size}} \left\{ \frac{\text{cfu}}{\text{ml}} \right\}$$

2.3 Screening of Proteolytic Isolates
Skim milk agar was prepared and the above colonies were streaked on milk agar plates for testing the proteolytic activity of the organism. Isolates were inoculated onto plates

and incubated at 37°C for 24 h. Strains producing clearing zones in this medium were selected [1].

2.4 MALDI-TOF Analysis of the Proteolytic Bacteria:

Strains that produced the largest clear zones on skim milk agar plates were further identified using MALDI-TOF by an instrument named MALDI Biotyper [3]. MALDI-TOF is a precise and rapid tool for bacterial identification. An isolated colony of each strain was mixed with matrix and added to MALDI-TOF project list. Software generated a spectrum, which was then instantly matched against reference library to give identification, with the help of the score value obtained.

2.5 Preparation of Inoculum

Inoculum was prepared [2]. 100 ml nutrient broth solution was prepared and sterilized at 121°C for 20 min. The medium was inoculated under aseptic conditions with bacteria. The broth culture was incubated for 14 hrs on a rotary shaker (150 rpm) at 30°C and was used for inoculating the production medium.

2.6 Degradation of Chicken Feather by the Isolated Bacteria

For studying the biodegradation of keratinous material [2], the keratinous wastes (chicken feathers) were fragmented into pieces with about 1 cm long and added to the fermentation media as a sole source of carbon and nitrogen. These sources were added separately to the fermentation media at 1% w/v. The percent of keratinous waste degradation was determined.

2.7 Determination of Degree of Degradation (DD)

The residual feather was washed, dried and scaled to calculate DD [2] by using following equation-

$$DD\ (\%) = (TF\text{-}RF) \times 100/TF$$

Where, TF is the total feather and RF is the residual feather.

2.8 Partial Purification of Protease

The cell free extract from fermentation broth was partially purified by acetone precipitation method [5]. Protease was precipitated by prechilled acetone (30-80%) fractionation. The acetone was added to the cell free extract in 3:1 ratio and incubated for 60 min at -20 °C. The contents were subjected to centrifugation at 10000 rpm for 10 min. The supernatant was discarded carefully and the pellet was dissolved in Tris-acetate buffer (pH 7).

3. Results and Discussion

In the present paper, proteolytic bacteria isolated from tannery effluent were checked for their efficiency to degrade keratinous chicken feathers and the degree of degradation of feathers was evaluated. Tannery effluents are rich in organic waste. Keratin degraders are mostly found on keratinous substrates, hence tannery effluent was chosen for the study.

3.1 Isolation of Microorganisms from Lime Effluent and Screening of Proteolytic Isolates

The microbial load of the lime effluent from the tannery industry was determined by performing serial dilution. Out of numerous colonies observed, few distinct non-overlapping pigmented as well as non-pigmented transparent colonies were selected and subcultured regularly for further screening. Five strains, out of nineteen were able to form hydrolysis zones on skim milk agar plates. Out of the five strains, three strains exhibited vibrant clear zones of lysis, thereby indicating their potential of secreting extracellular protease enzyme that hydrolyzed the casein present in milk agar plates. Isolates – TI, T2 and T3 were able to form clear zones on the milk agar plates. Out of 3 isolates, isolate – T2 exhibited highest clear zone diameter of about 23 mm on 2.5% skim milk agar medium at pH 7.0 after 24 h incubation at 37 °C. Next to it, isolate – T1 exhibited 20 mm zone of clearance, followed by isolate - T3, which formed 15mm diameter of lysis zone.

Table 1: Zone diameter on skim milk agar plates of tannery bacterial isolates

S. No	Bacterial Isolate No.	Zone diameter (mm) after 24 h incubation
1.	T1	20
2.	T2	23
3.	T3	15

3.2 Maldi-Tof Analysis of the Proteolytic Bacteria

Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry was performed to identify the selected proteolytic bacteria. Based upon the score value, the organisms were identified as *Bacillus subtilis* (T1), *Bacillus flexus* (T2), and *Bacillus endophyticus* (T3). MALDI-TOF considers the protein profiles of the bacteria and accurately identifies the organism.

Table 2: Maldi-Tof identification results of the proteolytic isolates

Analyte ID	Organism (Best match)	Score value
T1	*Bacillus subtilis*	1.592
T2	*Bacillus flexus*	2.12
T3	*Bacillus endophyticus*	1.655

3.3 Degradation of Chicken Feather by the Isolated Proteolytic Bacteria

Inoculum was prepared and biodegradation of chicken feather was studied. Isolate T2 (*Bacillus flexus*) showed highest degree of degradation (68%) of chicken feather, when compared with other isolates. It hydrolyzed the keratin present in the feathers efficiently. Next to it, *Bacillus subtilis* exhibited 59% of feather degradation. *Bacillus endophyticus* showed moderate degradation of 47%. Results obtained were in accordance with the earlier reports of Harison *et al.*, 2014 [1]. who obtained 70% of degree of chicken feather degradation by employing *Bacillus licheniformis* and 35% of degree of chicken feather degradation by employing *Bacillus subtilis*.

Table 3: Bacterial strains showing feather degradation

Bacteria	Initial Weight (g)	Final Weight (After 4 Days) (g)	Degree of Degradation (%)
Control	1	1	0
TI-*Bacillus subtilis*	1	0.41	59
T2-*Bacillus flexus*	1	0.32	68
T3-*Bacillus endophyticus*	1	0.53	47

Degradation of chicken feather by the bacterial isolates

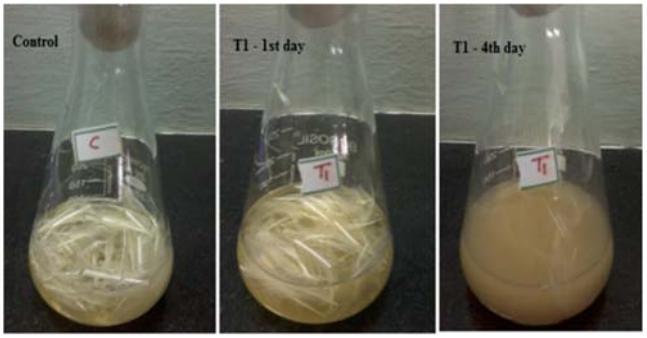

Fig 1: Degradation of chicken feather by *Bacillus subtilis* (T1)

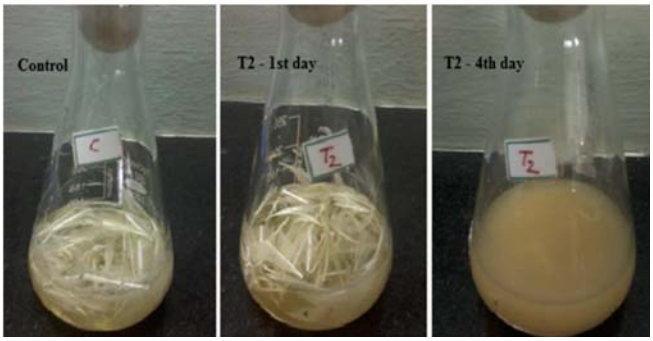

Fig 2: Degradation of chicken feather by *Bacillus flexus* (T2)

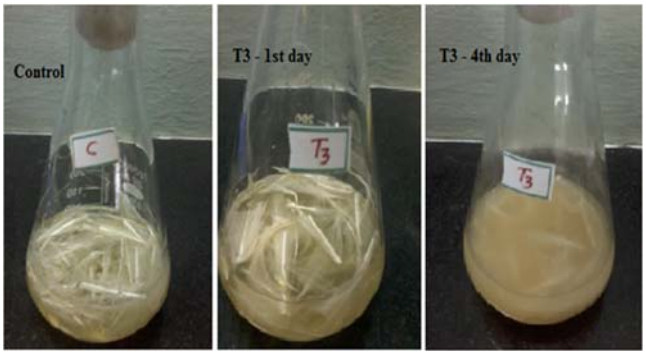

Fig 3: Degradation of chicken feather by *Bacillus endophyticus* (T3)

3.4 Partial Purification of Protease

Acetone precipitation was proved to be the best method of precipitating the protease enzymes from T1, T2 and T3 culture supernatants. The partially purified enzymes were stored carefully for further purification and characterization.

4. Conclusion

From the above results and discussion, it may be concluded and suggested that the microbial proteolytic enzymes could effectively degrade poultry feathers, than the conventional feather processing methods which are energy-intensive and expensive. Although microbial enzymes act as efficient keratin-degraders, increase in their yields and activity/stability has to be further optimized for commercialization and industrial application.

5. References

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