Isolation, Identification, Characterization of keratinolytic bacteria from poultry waste

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Abstract : Feathers contribute for 5-7% of chicken's overall mass and of their recalcitrant existence, have been one of the main contaminants. A decent provider of minerals, amino acids, and peptides to be used as fertilizer may be a feather made up of 90 percent keratin. Keratins are tough fibrous proteins, not soluble in organic solvents and water, frequently gathered in nature and major skin, hair, hoof, feather, nail, horn, etc. components, and keratin-degrading microorganisms like archaea, bacteria, fungi, and actinomycetes, use keratinases to attack keratin. Some microbes have a keratinase enzyme used to degrade the keratin found in poultry waste. As conventional feather deterioration strategies absorb vast quantities of energy and reduce the overall consistency of the proteins, this offers a good choice for the management of poultry. Deterioration of keratin by keratinolytic bacteria can, therefore, be an option to the production of eco-friendly, cost-effective, inexpensive, and good source for minerals and nitrogen (N) as possible organic fertilizers. Degraded keratin has a lot of applications in various other fields like, detergent industry, leather industry, etc. This review focuses on properties of keratinases, different types of keratinases, and different isolation methods for the diagnosis and characterization of keratinolytic bacteria obtained from the poultry waste.

Index Terms - Feather, Keratin, Keratinases, Keratinolytic bacteria, Metallo-protease, Poultry waste

I. INTRODUCTION

Feathers are generated in large quantities in poultry farms as a discarded by-product, approaching thousands of tons globally every year [1]. Feathers constitute 5 to 7 percent of a mature chicken's overall mass. Because of inadequate of poultry trash mostly feathers, has been one of the key contaminants because of its obstinate behavior [2]. They will serve as reservoirs for the maintenance of several microbes that are harmful to humans, like Salmonella and Vibrio. It also releases different toxins, such as mercury, nitrous oxide, hydrogen sulfide, negatively influencing public wellbeing and the climate. This are currently either dumped into landfills or incinerated in a generator furnace for power plants. While land application is an alternative, prolonged usage of both harmful chemicals and microorganisms will contribute to severe high levels of soil nitrogen with run-off contaminating streams and ground water. The existing important application of feather is its processing into feed meal/animal flour after high temperature processing and milling and used as a protein substitute in domestic livestock feed mixtures. In terms of overall cysteine, valine, and threonine quality, feather meal is comparatively low-cost and has been shown to be valuable to soybean meal [3]. Feather meal processing, though, is a costly method that kills many amino acids, creating a commodity of low intestinal absorption and varying nutritional content. An appealing option to enhancing the nutritional benefit of feather waste is complex hydrolysis by microorganisms that have keratinolytic activity. Keratinolytic microbes and enzymes produced by them may be used to increase the digestibility of keratin present in feather with major uses in the production of waste keratin from the poultry industry and the nutritional enhancement of feather meal that could substitute as much as 7% of increasing chicks' dietary protein [4]. Feather hydrolysates developed by microbial keratinase were used as livestock feed supplements. Moreover, keratin hydrolysates are theoretically used as unusual amino acids, edible film processing, and agricultural fertilizers [5]. In the meat, pharmaceutical sectors, detergent, pesticide, and leather, microbial keratinolytic enzymes have uses and, over all else, they increase the digestibility of keratin materials like feather feed.

II. KERATIN

Keratins are the major constituents of feathers. Keratins are non - soluble proteins present not just in feathers, but also in fur [6], skin, nails, horns, and other epithelial coverings. Due to the extremely stiff framework formed by comprehensive disulphide bonds and cross-linkages, keratin is resistant to degradation. Keratins are insoluble and resistant to the action of traditional proteolytic enzymes, like pepsin or trypsin, in weak acids, organic solvents, and water [7]. Feather keratin comprises essentially of α-helical and some conformations of the β-sheet. The exterior quill is constructed nearly exclusively of the β-sheet. Due to increased cystine content and, therefore, a far superior existence of disulphide (S-S) bonds that bind corresponding keratin subunits, β-sheet keratins are tougher than α-helix. Glutamine, cystine, proline, lysine, threonine, valine, and serine, are main components of keratins.

III. KERATINOLYTIC BACTERIA

Many bacterial strains which are suspected of deteriorating feathers are recognized. Enzymes that specifically deteriorate the β-keratin present in feathers are released by these strains of bacteria. For numerous bacteria species, like Vibrio [8], Thermoanaerobacter [9], Bacillus [10], Flavobacterium [11], Chryseobacterium [12], etc, keratinolytic action has been documented. Keratinases from several other strains of bacteria, including Lysobacter, Pseudomonas, Kocuria, Nesterenkonia, Stenotrophomonas, Microbacterium, Meiothermus [13], and Serratia were also identified. The organisms of Bacillus are big producers of keratinase.

Table 1 – Different bacteria's isolated by different researchers

No.	Bacteria isolated from poultry waste	Reference
1.	Bacillus licheniformis	Williams et al. (1990) [14]
2.	Xanthomonas maltophila	De Toni et al, (2002) [15]
3.	Chryseobacterium sp. kr6	Riffel al, (2003) [12]
4.	Microbacterium arborescens kr 10	Thys et al, (2004) [16]
5.	Burkholderia, Chryseobacterium, Pseudomonas sp.	Riffel and Brandelli, (2006) [17]
6.	B.megaterium F7-1	Park and Son, (2007) [18]
7.	B. cereus KB043	Nagal and Jain, (2010) [19]
8.	Nocardiopsis sp. SD5	Saha et al, (2012b) [20]

IV. BACTERIAL KERATINASE

Keratinases are unique proteolytic enzymes which can consume hardly soluble to non - soluble keratin, like feathers, nails, fur, and wool. Keratinases are usually serine proteases or metalloproteases which hydrolyze keratins. The enzymes are often distinguished by the strong particularity of the substrate for keratin. As they have wide substrate particularities for a number of proteinase K-resistant proteins, they remain apart from traditional proteases [21]. It is understood that a number of fungi, actinomycetes, and bacteria, develop keratinolytic enzymes. Actinomycetes, Bacillus spp., and streptomyces spp., are among the major keratinase producing bacteria in the gram-positive bacterial category. Most of them are popular in their biophysical and biochemical characteristics, keratinases demonstrate considerable variety [22]. Through examination of their protein sequences, some keratinases have been identified with the subtilisin family of serine-type proteases. Most keratinases are efficiently activated at the pH of 6 to 9 and temperature of 30-50 ° C, but at various pH and temperature, various bacterial species exhibit distinct behavior.

Table 2 -Various bacteria and their optimum activity

No.	Bacteria	Substrate	Active pH	Active temperature (°C)
1.	Bacillus spp., [23]	Azokeratin	5 to 9	30 to 40
2.	Chryseobacterium sp. kr6	Keratin azure	8	40-60
3.	*Bacillus licheniformis* ER-15	Feather, hooves	7 to 12	30-80
4.	Pseudomonas aeruginosa C11	Feather, collagen	5 to 10	60
5.	Microbacterium sp. kr10	Feather, casein, keratin	7.5	50
6.	Lysobacter NCIMB 9497	Keratin azure	-	50

Some properties of keratinases from various bacteria are mentioned below -

- The enzyme keratinase Q1 was purified, characterized, and isolated from Chryseobacterium sp. kr6 which is a member of the M14 metalloprotease family [24].
- The metalloproteases have also been shown to be able to destroy keratin derived from B. MTCC subtilis [25], Lysobacter NCIMB 9497 [26], and Streptomyces sp. 594 [27].
- The lowest molecular weight of the keratinase is developed by Bacillus pumilus A1 [28].
- The highest molecular weight keratinases are produced by K. rosea [29].

V. TYPES OF KERATINASES

- Serine keratinases - They are proteases in which serine amino corrosive is stabilized at the complex location of the reactant. Serine proteases depending on their composition are constructed. The serine protease portion depends on serine focused peptide bond nucleophilic attack. They are most active at alkaline pH with a range of 8-11.
- Metalloprotease - They are complementary proteases that involve metal molecules for the formation of their reactants. They are most active at neutral and slightly alkaline pH.

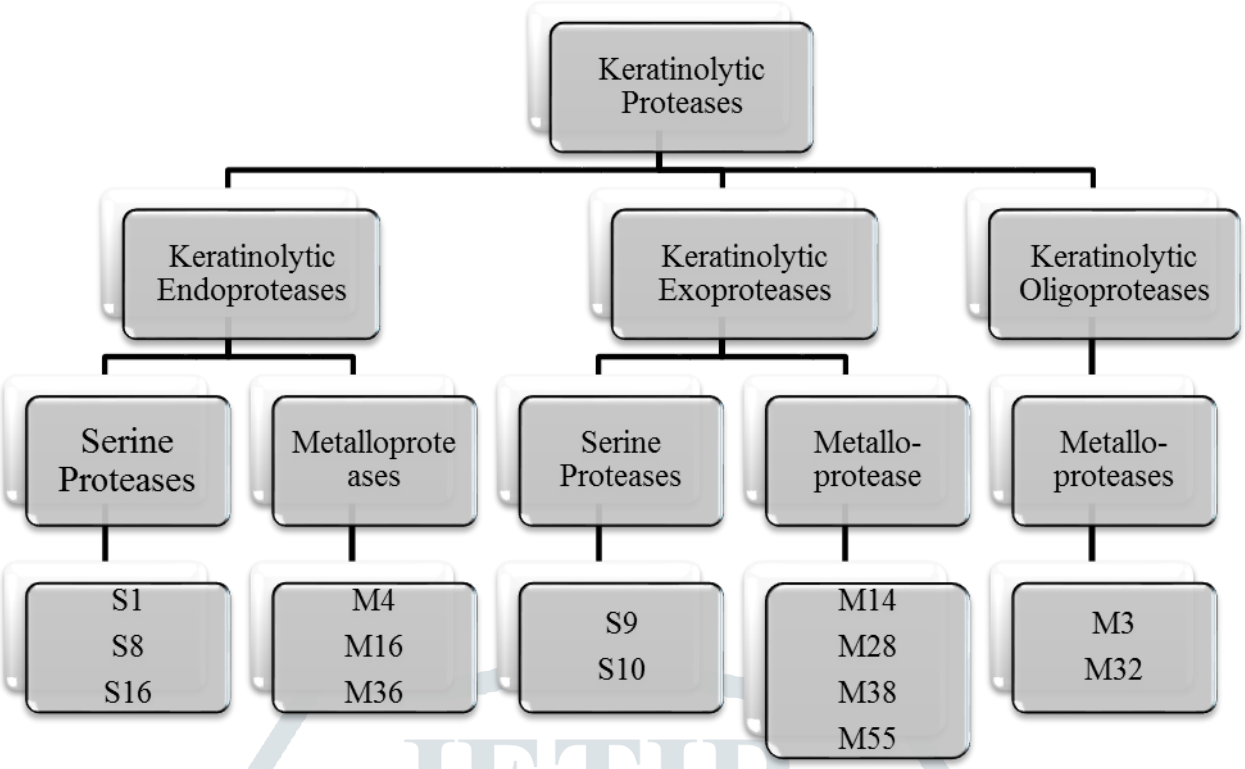

Figure 1 – Types of keratinases

VI. MECHANISM OF KERATIN DEGRADATION

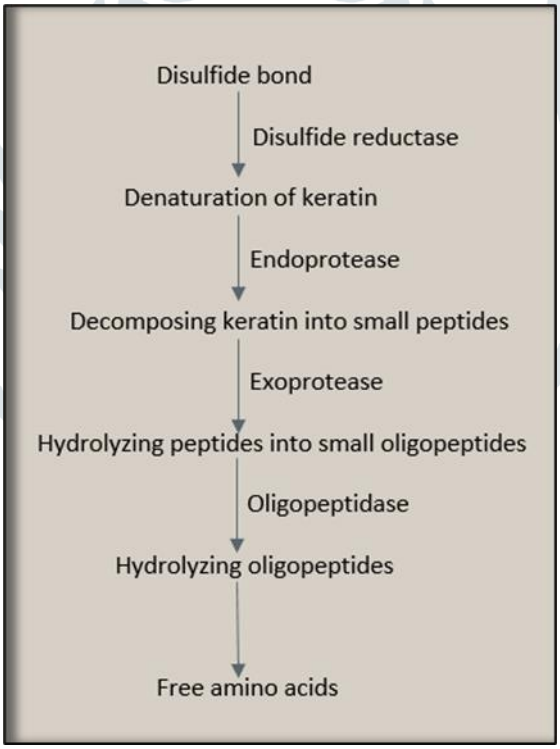

Figure 2 – Keratin degradation process by various keratinases [30]

The process of deterioration of feather keratin by keratinolytic bacteria involves sulfitolysis (disulfide bond destruction) followed by keratinase proteolytic action. Keratin is deteriorated into amino acids by different keratinases, such as endo-keratinase, exo-keratinase, and oligo-keratinase. First, the disulphide bond is disrupted, and this stimulates keratin denaturation. This keratin is then split into tiny peptides and the amino acids are then collected, which are then further used as needed.

VII. APPLICATION OF KERATINASES

- Feather meal as feed

Feathers are more than 90 percent protein, with β-keratin, a fibrous and insoluble structural protein that is heavily cross-linked by disulfide, hydrophobic, and hydrogen attachments, being the key ingredient. Onifade et al. [31] have thoroughly reviewed the usage of keratinases/keratinolytic microbes for feather improvement as poultry feed. In comparison, feather meal/raw feather hydrolysis utilizing keratinolytic microbes will attain dietary benefit.

- Waste management

Poultry production and processing generate a huge amount of keratinous wastes every year. The keratinases produced by the microbes are useful in management of these wastes by recycling it into something useful i.e., animal feed or other use.

- Feather meal as fertilizer

The protein-rich feather feed isolate produced for poultry feed can be used as a semi-slow-release N fertilizer for organic farming [32]. The N-rich feather feed (15 percent N), an affordable and readily accessible supply, provides as a possible replacement for guano. Not only it provides plants with N and stimulates microbial development, but also constructs the soil and improves the potential for water persistence. Thanks to its strong nutritional value, quick processing, and economic viability, the microbially hydrolyzed feather meal will also edge over the boiled feed as fertilizer.

- In detergent industry

Since ancient times, proteolytic enzymes have controlled the detergent market. Keratinases have the potential to connect and hydrolyze feather-like firm substances. As they are needed to operate on protein substrates connected to solid substances it is an essential characteristic of detergent enzymes, rendering them desirable ingredients for hard-surface cleaners. These can also assist in eliminating keratinous soils which are frequently present in laundry facilities, like collars of the shirts, where most proteases refuse to function [33].

- In leather industry

A variety of processes include leather refining techniques of which pre-tanning leads to the largest extent of contamination (approx., 70 percent). For the dehairing phase, keratinolytic proteases deficient collagenolytic and possessing moderate elastolytic behaviors are progressively being studied. They can aid in the targeted degradation of the keratin material in the follicle, extracting preserved hair without compromising the leather's mechanical properties [34].

- Other medical uses

Medical keratinase-based therapies have demonstrated considerable effectiveness in treating acne. The predominant cause behind certain instances is the production of unnecessary keratin that inhibits the sebaceous glands. The keratinase efficacy allows them the opportunity to remove the wound scar.

Prions are amongst the serious contagious proteins that cause brain disorders that are contagious and lethal. Feather decomposition keratinases have the ability to hydrolyze the β-keratin framework; they may therefore destroy the prion protein [35].

It is possible to describe hyperkeratosis as irregular thickenings of the outer skin plate, which is called corn and calluses. The key part of keratin is these shaped materials; hence, dermatologists recommend different developed keratinase-based medicines to hydrolyze those rigorous matters.

VIII. VARIOUS CHARACTERIZATION METHODS

1. Morphological characterization
   - Colony morphology
   - Cell morphology
   - Gram staining
2. Biochemical characterization
   - Casein hydrolysis
   - Catalase activity
   - Citrate utilization
   - Gelatin hydrolysis
   - Starch hydrolysis
3. Physiological characterization
   - Endospore test
   - Motility
   - Growth at various temperatures and pH
4. Molecular characterization – sequence analysis of 16S rDNA of the bacteria.

IX. ISOLATION, IDENTIFICATION, AND CHARACTERIZATION OF KERATINOLYTIC BACTERIA

Table 1 – Identification, isolation, and characterization of different keratinolytic bacteria by different researchers

No	Isolation	Characterization	Identification	Author names
1.	Feather extracts were collected from 2 poultry in Dhaka, Bangladesh, to extract keratinolytic microbes. Specific requirements, including chicken feathers as a carbon source and N, were created. A gram of the sample was added to a primary enrichment flask (capacity of 500 ml) and incubated for 7 days at 37ºC in an orbital shaker. On 50 ml of the original growth medium, a single ml of growth media was added, and the sample was cultured three times on the same parameters. The ultimate broth was drained and delivered into clean tubes to remove firm molecules. On nutrient agar surfaces, diluted samples were cultured. At 37ºC, the plates were incubated.	Two isolates were obtained. They were spore-former, rod-shaped, and Gram positive. Both the isolates utilize glucose and sucrose, although not lactose. They were also negative for the MR-VP test, negative for oxidase, but positive for catalase. Citrate was not utilized by the organisms, and both were able to convert nitrate to nitrite.	Typical features of Bacillus sp., were seen by both isolates.	**Jahan et al. [36]**
2.	1gm of poultry waste was sequentially dissolved in order to minimize the original amount of microbes. Then the dilution was inoculated into the basal feather broth. Just before applied to the medium, feathers were cleaned, dried and hammer milled. Autoclaving was used to sterilize the medium. In a regulated environmental shaker, both incubations were conducted at 37 ° C with shaking at 120 rpm.	The bacterium was extremely motile and developed aerobically, firmly catalase positive, and Gram negative.	*Bacillus licheniformis* was the identified bacteria	**Joshi et al. [37]**
3.	Feathers were gathered from a number of local poultry industry locations. In peptone broth (5 g l–1), feathers were flooded and incubated at 30 °C for 24 hours. The suspension was used to streak plates of feather-meal agar (0.5 g NaCl, 10 g feather-meal, 15 g agar, 0.4 g KH2PO4, and 0.3 g K2HPO4) that were incubated for 3 days at 30 °C. In feather-meal agar plates, single colonies have been isolated and tested for their capacity to hydrolyze keratin. For further study, colonies creating clear zones in this medium were chosen.	The bacterium evolved aerobically, shaped standard yellow colonies, gram-negative, and straight rod.	Two genus Chryseobacterium and Flavobacterium were identified	**Riffle et al. [12]**
4.	Soil samples were obtained from poultry waste dumping sites Pre-enrichment was performed by moving 10 g of soil sample into 90 mL Feather Meal Broth (FMB) (0.3 g L-1 K2HPO4, 0.5 g L-1 NaCl, 10	Gram positive, occasionally chains-shaped rod, endospore formation, and optional anaerobic bacteria	*Bacillus cereus* was identified	**Reyes et al. [38]**

	g L-1 cut feathers, and 0.4 g L-1 KH2PO4) and afterwards incubated at 37 ^{0}C for 48 hours. The sample was then diluted serially at 10-3, 10-5 and 10-7 dilutions and incubated in Feather Meal Agar Plates for five days at 37 0C. There were distinct colonies stripped into FMA and incubated for two days.			
5.	In peptone broth (5 g L–1), feathers were flooded and incubated at 35 ° C for 24 hours. On feather meal agar plates comprising 0.5 g NaCl L-1-, 15 g agar L-1, 10 g feather meal L-1, 0.4 g KH2PO4 L-1,0.3 g K2HPO4L-1, and incubated at 35°C for 72 h, the suspension was streaked.	The staining characteristics showed that the bacterium is Gram positive, producing spores, and defining rods. Biochemical features like starch hydrolysis, catalase, H2S output, citrate utilization, casein hydrolysis, lactose acid (TSI test), oxidase test, gelatin hydrolysis, etc. were observed positive while MacConkey agar growth, indole test, Voges Proskauer test, methyl red test, nitrate reduction, glucose acid (TSI test), showed negative results for the isolated strain agar test.	*Bacillus subtilis* was identified	**Chhimpa et al. [39]**

X. FUTURE ASPECT

In the field of keratinase characterization and to address the processes of keratin degradation in nature, further experimental initiatives are required. The analysis of the structure of successful microbial communities and the explanation of the interactions between the members of the consortia can be driven by the models found for the decomposition of keratin in nature; this is a highly important area, both in terms of recognizing the transformation of biomass in nature and in improving the industrial upgrading of keratin waste and thus the utilization of energy. It is important to revisit and thoroughly define and appreciate the whole area of nutritional benefit for animals of protein-rich feed supplements made from keratinaceous waste. In the processes available today, just a limited portion of the keratin content is typically completely decomposed. It will be extremely advantageous to standardize and available, well-characterized samples to be used as standard for new developments of enzymes and enzyme blends. But more significantly, it is essential to thoroughly illustrate the nutritional benefit and promise of keratin-derived livestock feed [40]. There is a requirement for more analysis and testing on certain other forms of keratinase application. Enzymatic dehairing is gradually used as a safe solution to prevent the issue produced in tanneries by sulfide [41]. The benefits of enzymatic dehairing are the reduction of the amount of sulfide in the effluent, the regeneration of high-quality hair, and the removal of bate in the deliming. Due to their properties as extremely effective and selective catalysts, the scope for the industrial usage of enzymes in leather processing is substantial. For cosmetic and medicinal uses, these enzymes have desirable qualities where collagen cannot be targeted.

XI. CONCLUSION

A description of keratinase and keratinolytic bacteria is given in the current analysis. Owing to their capacity to function on the strong, stiff, unsolvable structural protein-keratin, keratinases are regarded as current-day proteases. Keratinases are important enzymes for keratin garbage bioprocessing. A remarkable property is their capacity to destroy the recalcitrant protein keratin. The extract with a strong keratinolytic ability may be used for biotechnological utilization in the keratin hydrolysis method for the processing of poultry waste for environmental conservation and for biomass conversion of feathers into livestock feed product. In different industrial procedures including keratin hydrolysis, the keratinolytic activity of the keratin degrading isolate would have biotechnological use. The usage of microbial keratinase is an advantageous and inexpensive solution to the treatment of keratinous waste and the avoidance of environmental contamination.

XII. ACKNOWLEDGMENT

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