SCREENING OF KERATINOLYTIC BACTERIA FROM FEATHER DUMPING SOIL

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Abstract: Feathers are byproduct waste of poultry processing plant and produced in large amount. Chicken feathers constitute about 90% protein of which the main component is beta-keratin, a fibrous and insoluble structural protein extensively cross linked by disulfide bonds. This renders them resistant to digestion by animal, insects and other proteases leading to serious disposal problems. A small percentage of feather waste is steamed, chemically treated, ground, to form feather meal a dietary protein supplement for animals. Alternatively, keratin can be biodegraded by some Keratinolytic bacteria and in this study Keratinase producing bacteria and their Keratinolytic enzyme production was investigated. This enzyme belongs to class hydrolase which are able to hydrolyze insoluble keratins more efficiently than other proteases. Fifteen (15) bacterial isolates from chicken feather waste dumped soil were screened for feather degradation by growing them with feathers as their primary source of C, N and energy. Two of the isolates designated as PS-D and PS-H showed higher keratinolytic activity were characterized and selected for further studies based on its superior keratinolytic activity. Fermentation using feather as a substrate was carried out on mineral salt media (MSM) for seven days which resulted in complete degradation of feather. The percentage of weight loss of feathers was checked after 3, 5 and 7 days. The optimum pH and temperature of the keratinase enzyme was also studied. Thus, the degraded feather wastes represent a potential alternative to expensive dietary ingredients for animal feedstuffs since they are treated biologically and the amino acids are also not destroyed which are usually destroyed by physical and chemical treatments.

Keywords: Feather, Keratin, Keratinase enzyme, degradation, Keratinolytic Bacteria.

Introduction: Worldwide, 25 billion chickens are killed annually and around 8.5 billion tonnes of poultry feathers are produced, amongst which India's contribution alone is 350 million tonnes. The poultry feathers are dumped, used for land filling, incinerated or buried, which involves problems in storage, handling, emissions control and ash disposal. Discarded feather also causes various human ailments including chlorosis, mycoplasmosis and fowl cholera [1].

Feather is pure keratin protein and is insoluble and hard to degrade due to highly rigid structure rendered by extensive disulphide bond and cross-linkages. The keratin chain is insoluble, high stable structure tightly packed in the "α-helix ("α-Keratin) and β -sheets (β - keratin) into super coiled polypeptide chain [2]. 90% of the feather contains β - keratin by mass and β - keratin are extensively cross linked [3]. Cross-linking of protein chains by cysteine bridges confers high mechanical stability and resistance to proteolytic degradation by pepsin, trypsin and papain. The disulphide bonds of β -keratin can be reduced by the enzyme disulphide reductase followed by proteolyitc keratinases [4],[5]. Feather can be utilized so that it can be used as animal feed, this can prevent accumulation of feather in the environment and decrease the development of pathogenic strains. Biotechnological processing of feathers for the

production of feather meal, instead of chemical processing is preferred as it preserves the essential amino acids (Methionine, Lysine, Histidine) [6].

Innovative solution for waste disposal along with biotechnological alternative for recycling of such wastes is of utmost importance. Structural keratin can be degraded by some proteolytic micro-organisms as reported by Onifade *et al* [3]. Keratinase are specific protease that degrades keratin specifically. It is produced by Saprophytic and Dermatophytic Fungi and some *Bacillus* species. Feather degrading bacteria are physiologically diverse and approximately 99% of Bacterial species are unculturable because of their ability to enter non culturable state or because no culture methods have been established [7]. A number of keratinolytic microorganisms have been reported, including some species of fungi such as *Microsporum Trichophyton* and from the bacteria *Bacillus*; and *Streptomyces*; and actinomycetes [8]-[17]. Increase in keratinolytic activity is also found to be associated with thermophilic organisms, which require high energy inputs to achieve maximum growth and the decomposition of keratin wastes [18].

Till date most of purified keratinases known cannot completely solubilize native keratin; their exact nature and uniqueness for keratinolysis is still not clear [19],[20]. There is always a requirement of isolation of enzymes from new sources to meet the industrial and environmental demand.

Keratinolytic enzymes have found important utilities in biotechnological processes involving keratin-containing wastes from poultry and leather industries, through the development of non-polluting processes. After hydrolysis, the feathers can be converted to feedstuffs, fertilizers, glues, films and as the source of rare amino acids, such as serine, cysteine and proline [5], [10], [21]. The present study is concerned on the isolation and identification of bacteria from feather dumping soil, Takka, Old Panvel, Navi Mumbai, Maharastra showing keratinolytic activity.

Materials and Methods:

The chemicals used during project work are of pure grade, produced from Hi-Media Pvt. Ltds. Mumbai. The glassware used were of Borosil, all the glassware used for practical purpose was first clean with acid and rinsed with double distilled water.

Isolation and Primary screening of keratinase producing Bacteria:

The soil sample was collected from the feather dumping sites in Takka, Old Panvel in a sterile polythene bags. Serial dilution of the collected soil sample was prepared by adding 1 g of the soil sample to 9 ml of sterile saline. Then serial dilution up to 10^{-9} was done using sterile saline. All the dilutions were plated on Casein Agar medium for testing the caseinolytic activity of the organisms and incubated at 37°C for 5 days and the plates were examined for clear zone formation on the agar plates.

Secondary Screening of Keratinolytic bacteria:

The colonies obtained from casein agar plate were inoculated in 100ml of Mineral Salt Medium (MSM) with 1gm chicken feathers as a source of carbon and nitrogen were incubated under shaker conditions to check for feather degradation. The cultures which showed the maximum extent of feather degradation with minimum duration were selected for further studies. Feather degradation was confirmed visually.

Substrate:

Chicken feathers collected were used as substrate. Feathers were washed with distilled water and subsequently dried in hot air oven at

50°C. After this, pre treatment was done by keeping these feathers in chloroform: methanol (1:1) solution for 48 hrs. These feathers were again dried at 40°C and then stored at 4°C for further use.

Morphological, biochemical and molecular identification of the keratinolytic bacterial strain:

The morphological and biochemical characteristics of the isolated bacteria were studied according to Bergey's Manual of Systematic Bacteriology [22]. Further identification of the microorganism was performed through 16S rDNA sequencing. The 16S rDNA was amplified by PCR using a DNA thermal cycler (**PEQ-LAB**).

16S rRNA sequencing approach and phylogenetic analysis:

A PCR was performed in order to identify the 16S ribosomal (rDNA) of the isolates. The primers used were forward Univ16F **(AGAGTTTGATCMTGGCTCAG)** and reverse primers Univ16R **(TACGGYTACCTTGTTACGACTT).** The PCR products were further used for 16S rRNA sequencing. Sequence similarity search was made for the sequence of PS-D using BLAST search (NCBI) and the ribosomal database project. Evolutionary tree was inferred by using the neighbor joining method. The CLUSTAL W and W2 programmes were used for multiple sequence alignment and phylogenetic analysis.

Enzyme Assay:

Preparation of keratin solution:

Soluble keratin was prepared from white chicken feathers by the method of Wawrzkiewicz. 10 gm chicken feathers in 500 ml Dimethyl Sulfoxide (DMSO) were heated in hot air oven at 1000 C for 2 hrs. Soluble keratin was then precipitated by addition of cold acetone (1 L) which was previously kept in freezer for three days. After, addition of cold acetone; container was kept in deep freezer for two hours followed by centrifugation at 10,000 x g for 10 minutes. The resulting precipitate was washed twice with distilled water and dried at 40°C. One gram precipitate was dissolved in 10mM Tris HCl buffer pH 9.0 and preserved as a stock solution.

Keratinase activity assay:

The keratinolytic activity was assayed with 1.0 ml of crude enzyme by adding in it 1.0 ml of keratin solution. This was kept at 50°C in a water bath for 10 minutes and the reaction was stopped by adding 2.0 ml of 0.4 N Trichloro Acetic Acid (TCA). After centrifugation at 1450 x g for 30 minutes, the absorbance of the supernatant was determined at 280 nm against a control. The control was prepared by incubating the enzyme solution with 2.0 ml TCA without the addition of keratin solution.

Characterization of crude enzyme:

Effect of pH:

The optimum pH of the crude enzyme was found by dissolving the keratin at various buffers using 0.05 mol/L acetate buffer (pH 4-5.5), 0.05 mol/L phosphate buffer (pH 6-7.5), 0.05 mol/L Tris-HCl (pH 8.0 - 9.0). Based on the above mentioned pH the keratin solution was prepared for various buffers. The experiment on the effect of pH on enzyme stability was carried out by incubating the enzyme solution at pH ranges of 4.0 – 9.0. Then the enzyme activity was determined by the standard enzyme assay.

Effect of temperature: With the optimum pH of the crude enzyme as constant the optimum temperature was found by incubating the enzyme with the substrate (keratin solution prepared according to optimum pH) at varying temperature range from 30°C to 80°C. The experiment on the effect of temperature on enzyme stability was carried out by incubating the enzyme solution at temperature ranges of 30°C -80°C Then the enzyme activity was determined by the standard enzyme assay.

Determination of feather degradation: Residual feathers were harvested from the fermentation media by filtering it over whatman filter paper No.3. The harvested feathers were kept in hot air oven at 50°C until weight stabilized to constant value. The difference between the weight of residual feather obtained from the control and that of inoculated media has been used as measure of feather degradation. Degradation was expressed in percentage.

The percentage of degradation of feather by the two bacterial isolates was determined by calculating the percentage of weight loss of feather. The percentage of weight loss was calculated by the following formula:

Percentage of weight loss = [Initial weight – Final weight x 100]/Initial weight

Results:

Screening of isolates for feather degradation:

The result of the screening test of the isolates on feathers (Table 1) showed that two (2) of the fifteen (15) bacterial isolates recovered from the sample were able to grow on the whole chicken feather as its primary source of carbon, nitrogen and energy, but the degree of degradation varied with each isolate after 7 days of incubation at (32°C). The isolates were designated PS-A, PS-B, PS-C, PS-D, PS-E, PS-F, PS-G, and PS-H…PS-O (PS- Panvel Soil). Extent of feather degradation was found maximum within seven days in media inoculated with isolates PS-D and PS-H in comparison to other isolates. All the Isolates were able to grow in minimal medium with father as a sole C and N source. The isolates displayed some breakdown of the feather. But their degrading ability was slow and took longer duration as compared to PS – D and PS – H. The isolates PS-D and PS-H degraded all the feather parts like vanes, shafts and quill within seven days. (Fig.1)

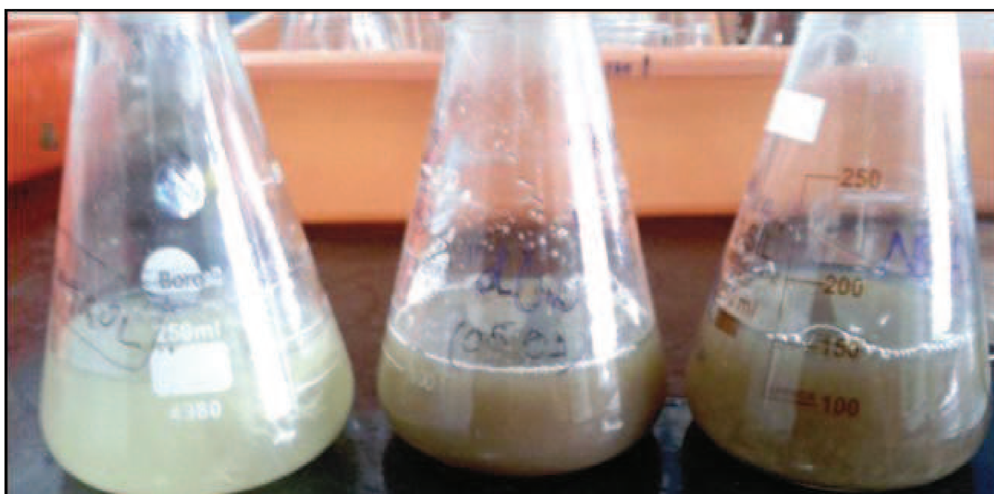
Characterization of the feather-degrading strains:

The isolates PS-D and PS-H which showed the most degradation of feather parts: vanes, shafts and quill within seven days were characterized. The identification of the keratinolytic bacteria was based on cell morphology, colony morphology, and several biochemical tests (Table 2 and 3). Isolates PS-D and PS-H were determined to be Gram negative short rods, and fluorescence organisms. The isolate PS - H formed purplish pink colonies and PS-D showed greenish blue colored colony on agar plate (Fig.2). The organisms were aerobic, motile, and oxidase and catalase positive. Additional morphological, physiological and biochemical tests were conducted as shown in (Table 3). The features agreed with description of Bergey's Manual of systematic Bacteriology [22]. These results suggested that these two strains belong to genus *Pseudomonas.* On the basis of morphological, cultural characteristics and 16s rRNA studies the strain PS-D was found to be *Pseudomonas aeruginosa* having highest homology (96%) with *Pseudomonas aeruginosa.* From these results, PS-D was identified as a new strain of the species *Pseudomonas aeruginosa.* 16S rRNA gene sequence of strain PS – D was submitted to GenBank and given accession number **KF613155-KF613156**. However PS-H did not give apt sequencing result which will be further attempted individually.

Table.1. Feather degradation by isolates

S/N	Isolate designation	Extents of feather degradation
1	PS – A	+
2	PS – B	++
3	PS – C	+
4	**PS – D**	++++
5	PS – E	+
6	PS – F	+
7	PS – G	+
8	**PS – H**	++++
9	PS – I	++
10	PS – J	+
11	PS – K	+
12	PS – L	++
13	PS – M	+
14	PS – N	+
15	PS – O	++

++ = Degradation of feather vanes
++++ = Extensive degradation of feather vanes plus shaft degradation



Control PS-H PS-D

Fig.1. Feather degradation in MSM flasks

Table 2: Results of morphological and cultural characteristic of isolated bacterial strains

Colony Characteristics	PS –D	PS - H
Size	2mm	3mm
Shape	Circular	Irregular
Pigment	Shiny, Smooth	Shiny, smooth
Margin	Opaque	Opaque
Elevation	Raised	Raised
Consistency	Moist	Moist
Motility	Motile	Motile
Fluorescence	Positive	Positive
Microscopic Character		
Gram Stain	Gram Negative rods	Gram Negative rods

Fig.2. Growth of isolated bacterial strains on NA slant

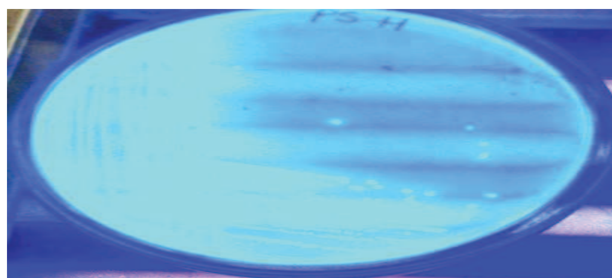


Fig.3. Fluorescence property of the isolates

Table3. Results of Biochemical Tests

Biochemical characters	PS-D	PS-H
Glucose: Aerobic	Negative	Negative
Anaerobic	Negative	Negative
Sucrose: Aerobic	Negative	Negative
Anaerobic	Negative	Negative
Lactose : Aerobic	Negative	Negative
Anaerobic	Negative	Negative
Mannitol: Aerobic	Negative	Negative
Anaerobic	Negative	Negative
Fructose : Aerobic	Negative	Negative
Anaerobic	Negative	Negative
Catalase	**Positive**	**Positive**
Nitrate	Negative	Negative
Urease	Negative	Negative
Oxidase	**Positive**	**Positive**
Gelatinase	**Positive**	**Positive**
Indole	Negative	Negative
Mthyl Red	Negative	Negative
Voges Proskauer	Negative	Negative
Citrate	**Positive**	Positive
Hughliefson	**Positive**	Positive
Triple Sugar Iron (TSI)		
Slant	Alkaline	Alkaline
Butt	Alkaline	Alkaline
H_2S	Negative	Negative
Gas	Negative	Negative
Identification of organism	***Pseudomonas spp***	***Pseudomonas spp***

Characterization of crude keratinase: The strains PS-D and PS-H grew well and completely degraded poultry feather in the mineral salt medium. The intense feather degradation was achieved in 50°C and initial pH adjusted to 7.0 and 8.0. Similar growth curve were observed with in this range of temperature (30°C, 40°C, 50°C, 60°C, 70°C and 80°C) and pH (4.0 – 9.0).

The keratinase was active in neutral and alkaline condition with an optimum activity at pH 7.0 as shown in (Figure 4.) depending on the buffer used. The enzyme was stable over a wide range of pH values, with the highest stability at pH 6.0 – 9.0 for 30 minutes as shown in (Figure 4.) The enzyme had an optimum activity at the temperature of 50°C for 30 minutes and the decrease in activity was observed with increase in temperatures for both the organisms. The enzyme was unstable at high temperatures but was stable at moderate temperatures as shown in (Figure 5.)

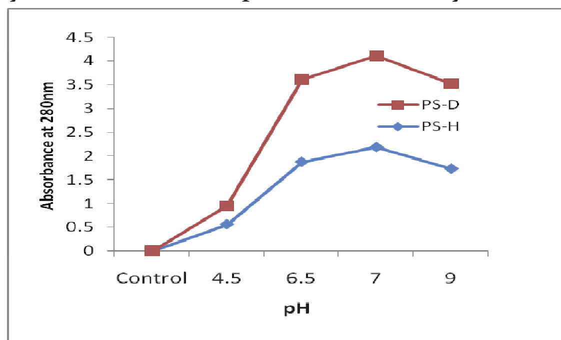


Fig.4. Effect of pH on crude Keratinase Enzyme activity

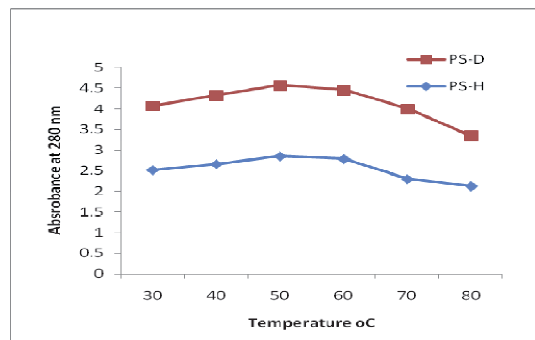


Fig.5.Effect of temperature on crude keratinase activity

Determination of percentage of feather degradation by PS-H and PS-D:

Residual feathers were filtered by (Whatman filter paper 3) and dried in hot air oven at 50°C until weight stabilized to constant value. The complete feather degradation was observed after 7 days of inoculation. 60% feather degradation was obtained after five days of incubation in PS-H and nearly 95% of degradation observed in seven days. Whereas more than 70% degradation was obtained in five days and nearly 100% degradation was observed after seven days of incubation in PS-D. It was very clear that feather degradation was maximum in sample subjected to long time incubation (Fig. 6 and 7).

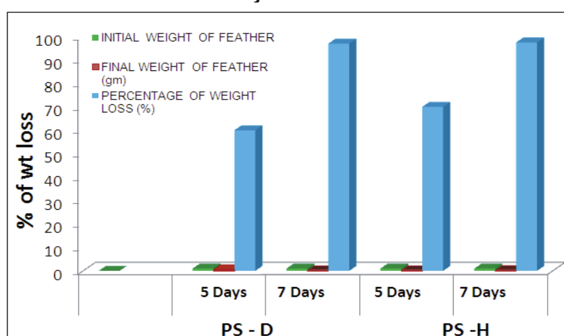


Fig.6. Percentage of feather degradation by PS – D and PS - H

Fig.7. Recovers (feather pieces) after degradation

Discussion: The microbial conversion of feather wastes is a potential technique for the degradation of feathers and their utilization as a feedstuff [6], [23]. Microorganisms that degrade feathers and their keratinolytic enzymes could be used to enhance the digestibility of feather keratin [24]-[26]. Many *Bacillus* species have been reported to produce keratinolytic proteases [24]-[29]. As well known, keratin degradation is mostly performed by gram-positive bacteria (Gupta *et al.*, 2006), although there are a few reports on feather-degrading gram-negative

bacteria [30],[31].

Bacteria were isolated from chicken feather waste dumped soil and they were found to be capable of hydrolyzing feathers. This shows that these isolates owned keratinolytic activity and ability to degrade keratin. Preliminary identification tests indicated that the isolates PS-D and PS-H were *Pseudomonas* species which was further confirmed by 16SrDNA analysis. The bacterial isolates used in the study are essentially aerobic. After culturing in shake flasks at 32°C, the feathers were degraded completely by PS-D and PS-H within seven days. Fast processing is critical for industrial applications such as in feed, fertilizer, detergent, leather and pharmaceutical industries.

The keratinase activity was observed over a wide range of temperatures 30°C to 80°C and pH in the range of 4.0 - 8.0 and the optimum activity of the enzyme was found at 50°C and at pH 7.0. In addition, the keratinase was stable over a pH of 6.0 – 8.0 and excellent thermostability at 50°C-60°C for 30 minutes. According to previous studies *Bacillus* species degrade native feathers completely when cultured at 50°C for 10 days and presents optimum growth and keratinase production at 50°C [1],[27],[28]. However data on feather degrading *Pseudomonas* species are fairly uncommon.

In our investigation, a new keratinase producing bacteria PS-D and PS-H were isolated. Complete feather degradation was achieved within seven days. Change in the colour of the MSM was observed which may be due to the increase in pH. Increase in pH is a significant indicator of strong keratinolytic activity during cultivation. Organisms with higher keratinase activity turned the media more alkaline than those showing lower keratinolytic activity. This may be due to the deamination of peptides and amino acids that are produced by degradation of keratin.

On the basis of its morphological and biochemical characteristics and 16S (rDNA) analysis, the strain PS-D was identified as a new strain of *Pseudomonas aeruginosa,* whereas further study is required for PS-H to confirm it as a *Pseudomonas* species. From the above results, we conclude that the isolate PS-D belongs to a new strain of *Pseudomonas* family and can secrete a large amount of keratinase and is more thermostable and has broader pH resistance. The keratinase from the two isolates degraded feathers effectively. Therefore, the newly isolated bacteria shows potential for use in biotechnological process that involve keratin hydrolysis and it can be expected to improve the nutritional value of feathers and other keratins and waste from poultry processing. *P. aeruginosa* is an opportunistic pathogen and can produce many toxic substances during fermentation, so the protease gene should been heterologously expressed in a more safe and efficient host. Further work on cloning and expression of recombinant protease from *P. aeruginosa* will been carried out in our laboratory.

Conclusion: Bioconversion of feather with *Pseudomona spp.* has great potential to protect our environment. This novel keratinolytic isolates could be a potential candidate for the degradation of feather keratin and also in de-hairing process in leather industry and can be used as additives in poultry field.

Future prospects: Since, feather protein has been shown to be an excellent source of metabolizable protein, these keratinolytic strains could be used to produce animal feed protein. Thus, the degraded feather wastes represent a potential alternative to expensive dietary ingredients for animal feedstuffs since they are treated biologically and the amino acids are also not destroyed which are usually destroyed by physical and chemical treatments. Utilization of these potential keratin degraders will definitely

find biotechnological use in various industrial processes involving keratin hydrolysis. Thus, the problem of poultry waste disposal would be solved to a large extent in an environment friendly manner.

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