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A novel feather degrading *Acinetobacter sp.* PD 12 isolated from feather waste dumping site in Mumbai

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Abstract:

Current methods of feather waste disposal include land filling, incineration or conversion to feather meal by steam pressure cooking. These methods require high-energy input and/or cause problems in storage, handling, emissions control and ash disposal. Discarded feather also causes various human ailments including chlorosis, mycoplasmosis and fowl cholera. Biodegradation of feathers using keratinolytic microorganisms represents an efficient alternative way to manage feather waste that could be coupled with production of valuable products such as protein hydrolysates and proteolytic enzymes. A new feather degrading bacterium was isolated from a local feather waste dumping site and identified as Acinetobacter sp. PD 12 based on 16S rDNA phylogenetic analysis. Aerobic cultivation of the isolate in minimal medium containing feathers at 32^{0}C for 6 days resulted in 70 % degradation of intact feathers. Scanning electron microscopy studies showed degradation of feathers by keratinase. The keratinase enzyme from the isolate was partially purified by ammonium sulphate precipitation followed by dialysis and characterized. Enzyme activity of partially purified enzyme was

determined as 63.72 units/ml using keratin azure as substrate. Optimum temperature and pH for keratinolytic activity were determined to be 32^{0}C and pH 8.0 respectively. The isolate shows promise for use in biological methods of feather waste disposal which are eco-friendly, safe and efficient as compared to currently used methods.

Key words: Keratinase, *Acinetobacter sp.* PD 12, protein hydrolysates, waste disposal, feather meal

Introduction:

Indian Poultry Industry is emerging as the world's second largest market and growing at a phenomenal rate of 12 to 15% every year. (Agrahari et. al. 2010; The Ministry of Food Processing Industries, India). India produces 1,400 million chickens a year, which is close to 27 million a week. Worldwide, 24 billion chickens are killed annually and around 8.5 billion tonnes of poultry feathers are produced. India's contribution alone is 350 million tonnes per year (Agrahari Sarit and Wadhwa Neeraj, 2010). Feathers represent 5 – 7% of the body weight of the chicken. Thus, enormous amount of poultry waste, especially feather waste is generated daily. The increased amount of feather waste generated by commercial poultry processing represents a pollution problem and needs adequate management. Currently, feathers are either converted to feather meal by steam pressure cooking, which requires high-energy input, or the 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 (Xu B et. al., 2009).

Keratin is the insoluble structural protein of feathers and makes up more than 90% of the total weight of the feathers and is known for its high stability. Keratin-rich wastes are

often difficult to degrade, since the polypeptide is densely packed and strongly stabilized by several hydrogen bonds and hydrophobic interactions. Despite elevated resistance of keratins to degradation, they do not accumulate in nature and can be efficiently hydrolysed by keratinolytic enzymes produced by a multitude of bacterial and fungi. Bacillus appears to be one of the prominent keratinolytic microorganisms. Although these organisms have been usually isolated from poultry waste dumping sites, they are ubiquitous in nature thriving under diverse ecological and environmental conditions (Gupta R. and Ramnani P., 2006). Keratinolytic enzymes are largely inducible and produced in the presence of keratinous substrates such as hair, feather, wool, nail, hairs, horn etc.

Biodegradation of feathers using keratinolytic microorganisms is attracting many research groups as it may provide an efficient way to manage feather waste that could be coupled with production of valuable products (Brandelli, 2008). The aim of this study was to isolate and identify new feather degrading bacteria from poultry waste dumping sites in Chembur region, Mumbai, Maharashtra.

Materials and methods:

1) Isolation of keratinolytic microorganisms:

i) Sample collection and primary screening: Rotted feathers and soil samples were collected from feather waste dumping sites in Chembur region, Mumbai, Maharashtra in sterile polythene bags. 0.1 gm of sample was added to 10 ml of sterile saline (0.85% NaCl) and vortexed thoroughly for 5 minutes. The suspension obtained was streaked on sterile Nutrient agar plates. Plates were incubated at 30^0C for 48 hours. Well grown isolated colonies were further purified and spot inoculated on sterile Milk agar plates. Colonies showing large and clear zone of

casein hydrolysis after incubation at 30^0C for 48 hours were selected and screened further for their keratinolytic potential.

ii) **Secondary screening:** The isolates obtained from primary screening on milk agar plates were inoculated in sterile Feather Meal Medium (FMM) having following composition (gm/l): NH_4Cl (0.5), NaCl (0.5), KH_2PO_4 (0.4), K_2HPO_4 (0.3), $MgCl_2$ (0.1), Yeast Extract (0.1), Feathers (10.0), pH 7.5. 1 ml of culture suspension of each isolate (0.1 absorbance at 540 nm) was added to 100 ml of sterile FMM in 250 ml Erlenmeyer flask. The flasks were incubated at 30^0C on shaker for 6 days. Feather degradation was observed visually as well as by measurement of percentage reduction in dry weight of feathers as compared to that of control (uninoculated FMM). Isolates showing significant percentage degradation of feathers were selected for further studies. They were sub-cultured and stored on sterile nutrient agar slants and preserved in refrigerator.

2) Assay for keratinase activity:

Keratinase activity was measured by Sigma Aldrich's assay protocol, using keratin azure k 8500 as a substrate. The reaction mixture contained 20 mg of keratin azure, 0.1 ml distilled water (D/W) and 0.4 ml of enzyme sample obtained from isolates showing keratinolytic potential. The reaction mixture was incubated at 30^0C for 1 hr. The colour developed was measured qualitatively at 595 nm using Systronics UV – VIS Spectrophotometer 119. One unit of enzyme activity is defined as increase in absorbance by 0.01 units.

3) Standard Assay for Proteinase K (Standard keratinolytic enzyme):

Keratinase activity of Proteinase K (Sigma Aldrich) was measured by the assay protocol mentioned above, except that standard enzyme was used for the assay.

4) Identification of keratinolytic isolate:

Gram staining, motility and certain biochemical tests were performed to identify the isolate. The identification was confirmed using 16S rDNA sequence analysis. For 16S rDNA identification, Bacterial Genomic DNA was isolated using the InstaGeneTM Matrix Genomic DNA isolation kit. 20µl of the supernatant was used per 50 µl PCR reaction. Using 16S rDNA Universal primers gene fragment was amplified using MJ Research Peltier Thermal Cycler.

Primer name	Sequence details	Number of base
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTTACGACTT	22

The PCR product was sequenced using the 518F/800R primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). The 16S rDNA sequence was BLAST using NCBI blast similarity search tool.

5) Scanning electron microscopy (SEM):

Feather samples from inoculated broth and control were removed on the sixth day of incubation, washed with sterile D/W followed by washing twice with iso propyl alcohol, centrifuged at 5000 rpm for 5 minutes and dried at 45^0C. Samples were fixed on aluminium stubs using double sided carbon tape and coated with chromium using sputter coaters to make sample conductive. Samples were then examined using Nova NanoSEM.

6) Extraction and purification of keratinase:

A saline suspension of each isolates with O.D adjusted to 0.1 at 540 nm was inoculated into sterile FMM and incubated for 6 days in shaker condition at 30^0C. After incubation the culture broth was filtered. The undegraded feather mass was collected and dried to constant weight to determine percentage feather degradation. pH of culture supernatant was noted. An uninoculated flask was kept as a control. Keratinolytic activity in the crude culture supernatant was assayed using keratin azure assay protocol. The experiment was performed in triplicates. Further, remaining culture supernatant was subject to ammonium sulphate precipitation by adding solid ammonium sulphate (Merck) (60% saturation). The solution was kept at 4^0C overnight. The precipitate was collected by centrifugation using refrigerated centrifuge (Remi C-24BL) at 4^0C and 10000 rpm for 15 minutes. Precipitate obtained was dissolved in 20 ml of D/W and dialyzed in D/W with 7 to 8 changes for 24 hours. This was used a source of partially purified enzyme for determination of keratinase activity as well as characterization studies.

7) Determination of optimum pH for keratinase activity:

In order to determine the effect of the pH on keratinase activity, the keratinase assay using keratin azure was performed in presence of following buffers: Acetate buffer (pH 5 & 6), Phosphate buffer (pH 7) and Tris-HCl (pH 8, 9 & 10).

8) Determination of optimum temperature for keratinase activity:

In order to determine temperature optima, the keratinase assay was performed by incubating keratinase enzyme - Keratin azure substrate reaction mixture at different temperatures ranging from 20^0C to 55^0C.

9) Determination of effect of ions, chelator and reducing agent on keratinase activity:

Keratinase assay was performed in presence of 5mM of ions ($HgCl_2$, $FeCl_3$, $CuSO_4$, $CaCl_2$, $ZnSO_4$), chelator (EDTA), and reducing agent (ß-mercaptoethanol). Residual keratinase activity was determined.

10) Zymography:

Activity staining of purified keratinase was performed using 12% polyacrylamide gels containing 1% casein. After electrophoresis, the gels were washed twice with distilled water and incubated at room temperature overnight. After incubation, the gels were stained with 0.1% Coomassie Brilliant Blue R-250 for 30 minutes and then destained with methanol-glacial acetic acid-water (30:10:60) for around 4 hours.

Results and Discussion:

Primary screening yielded thirteen isolates which were differentiated on the basis of their cultural and morphological characteristics. Further, these isolates were subjected to secondary screening using milk agar plates. Three potential protease producing isolates were selected based on the size of zones of casein hydrolysis on milk agar plate. These isolates were tested for their feather degrading potential and keratinase activity in culture supernatants.

After inoculation of each isolate in sterile FMM broth and incubation at 30^0C on shaker for 6 days, visual observation showed that the feathers were degraded. This observation was further confirmed by a measured decrease in dry weight of feathers as compared to that of control (Table 1)

Table 1: Percentage feather degradation and keratinase activity of isolates

Isolate No	Residual feather dry weight (grams)	Feather degradation (%)	Keratinase activity (Units/ml)	Final pH of culture supernatant
FDI 1	0.527	45.4	20.1	8.5
FDI 2	**0.281**	**70**	**40.69**	**8.7**
FDI 3	0.49	49.1	22.6	8.5
Control Flask	0.981	0	0	7.5

Degradation of feather by Isolate FDI 2 in FMM demonstrated that it could utilize feathers as the only source of carbon, nitrogen, sulphur and energy for growth. Based on the residual feather dry weight it was concluded that Isolate FDI 2 had the best feather degradation potential. It was observed that only 30 % of initial feather weight remained after 6 days of cultivation. During the incubation, pH of feather broth increased from 7.5 to 8.7 after 6 days. The increase in culture medium pH during cultivation is an important indicator of keratin hydrolysis. In fact microorganisms with strong keratinolytic potential cause culture medium to turn more alkaline then those that are less keratinolytic (Kaul and Sumbali, 1997). Thus pH increase during cultivation of FDI 2 indicates good keratinolytic activity of the strain. Hence it was selected for further studies.

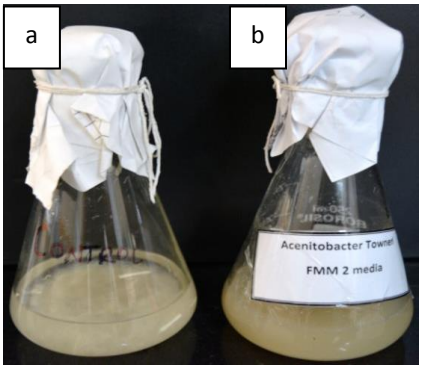

Fig1: a) Control flask: Uninoculated FMM broth; b) FMM inoculated with *Acinetobacter sp.* PD 12 after 6 days of incubation

Fig2: a) Residual feather after treatment with *Acinetobacter sp.* PD 12; b) Control – Untreated feathers

Keratinase activity of Proteinase K

Table 2: Keratinase activity in Units/ml using Standard Proteinase K (Sigma-Aldrich) as standard keratinase and Keratin azure k 8500 substrate, using keratinase assay protocol of Sigma-Aldrich.

Proteinase K activity (Units/ml)	Absorbance at 595 nm
25	0.14
50	0.27
75	0.35
100	0.49

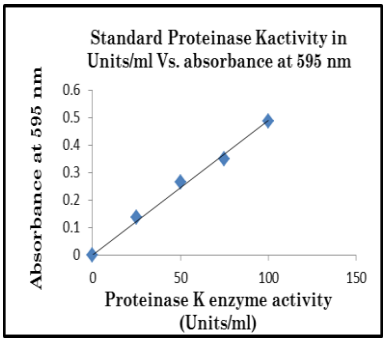

Fig. 3: Graph of keratinase activity using Standard Proteinase K as standard keratinase and Keratin azure k 8500 substrate.

16S rDNA sequence analysis

16S rDNA sequence analysis identified the isolate as *Acinetobacter sp.* PD 12

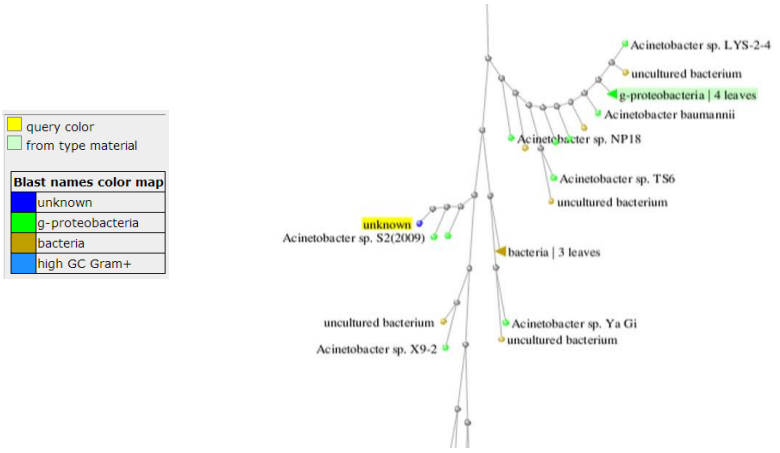

Fig 4: Phylogenetic tree showing the relationship of Query Isolate with *Acinetobacter sp.*

Scanning electron microscopy (SEM)

SEM was employed to examine structural changes during feather degradation. Controls are shown in Fig. 5 Control feathers show intact barbules and rachis. On the other hand, as seen in Fig. 6, feathers incubated with *Acinetobacter sp.* PD 12 for 6 days showed prominent surface erosion and extensive degradation of feather barbules with disintegration of rachis.

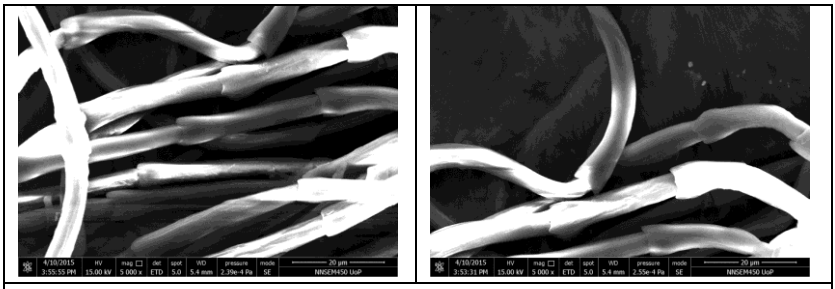

Fig 5: SEM of Control feather showing intact surface of barbules

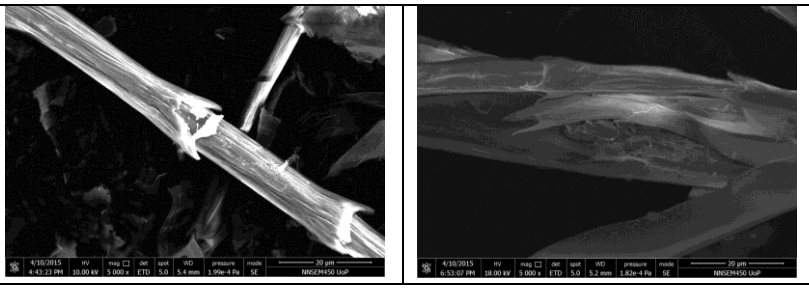

Fig 6: SEM of feather after treatment with *Acinetobacter sp.* PD 12 showing eroded surface of barbules

Keratinase activity form *Acinetobacter sp.* PD 12

Activity of crude keratinase enzyme and partially purified enzyme obtained from *Acinetobacter sp.* PD 12 was determined from standard Proteinase K graph. Activity of purified enzyme found to be 63.72 Units/ml as compared to crude enzyme whose activity was determined as 40.69 Units/ml (table 3). The increase in activity of purified enzyme can be correlated to increased concentration of enzyme due to purification and possibly may be due reduction in components that may affect enzyme activity in culture extracts.

Table 3: Keratinase activity in Units/ml of crude enzyme and partially purified enzyme

	Crude Enzyme	**Partially Purified Enzyme**
Absorbance at 595 nm	0.209	0.315
Activity in Units/ml as extrapolated from Standard Proteinase K graph	40.69	63.72

Optimum pH and temperature for keratinase activity

Optimum pH for keratinase activity was found to be pH 8.0 with 56.77 Units/ml (Fig. 6). As can be seen from the graph, enzyme also retained most of its activity in pH range of 7 to 10. These results are in accordance with previous reports, which have indicated that most keratinases are alkaline proteases

and are active in neutral to alkaline condition (Brandelli Adriano, 2008).

Effect of temperature on keratinase activity was determined in Tris-Cl buffer pH 8.0. The enzyme was found to be optimally active at 32^{0}C with 60.68 Units/ml of keratinase activity (Fig. 7). At 37^{0}C there was a slight decrease in enzyme activity to 59.16 Units/ml. At temperatures 20^{0}C and 55^{0}C, significant reduction in enzyme activity was observed.

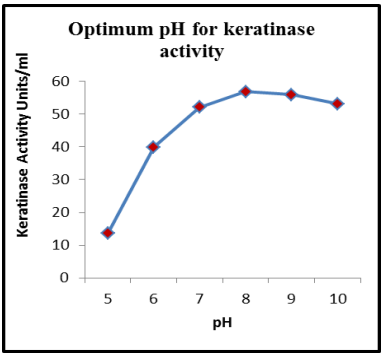

Fig. 6 Efffect of pH on keratinase activity

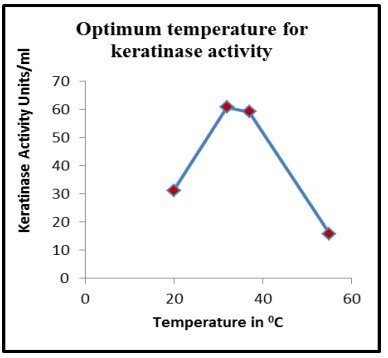

Fig. 7 Effect of Temp. on keratinase activity

Effect of ions, chelators and reducing agent on keratinase activity

As can be seen in table 4, Ca^{+2} increased the enzyme activity, whereas inhibition was observed with Fe^{+2}, Cu^{+2}, $Zn^{+2}$and Hg^{+2}. The enzyme activity was slightly inhibited by reducing agent ß-mercaptoethanol. Chelating agent EDTA, a metalloprotease inhibitor caused significant reduction in enzyme activity.

Table 4: Residual Keratinase activity in Units/ml in presence of ions, chelators and reducing agent

Ion / chelator /reducing agent	Concentration	Residual activity (%)
Control	--	100
$FeCl_3$,	5 mM	67.7
$HgCl_2$	5 mM	14.2
$CuSO_4$	5 mM	66

$ZnSO_4$	5 mM	27.1
$CaCl_2$	5 mM	114.9
EDTA	5 mM	29
ß-mercaptoethanol	5 mM	89.7

Zymography

Casein zymography was performed to determine presence of extracellular Keratinase in culture supernatants of *Acinetobacter sp.* PD 12. Fig. 8 shows a clear zone of casein hydrolysis confirming presence of significant quantity of extracellular Keratinase.

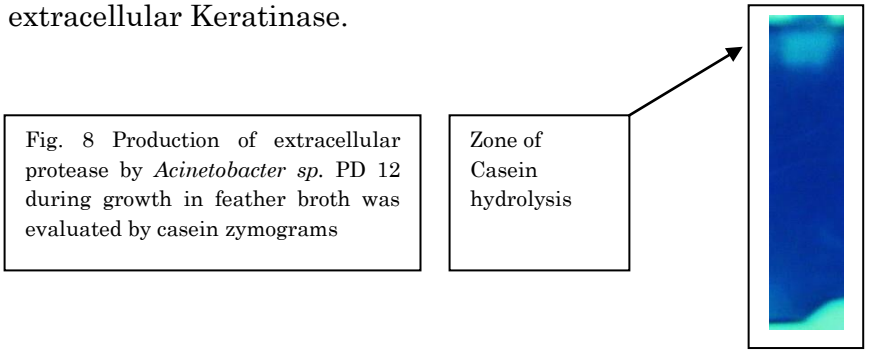

Fig. 8 Production of extracellular protease by *Acinetobacter sp.* PD 12 during growth in feather broth was evaluated by casein zymograms

Conclusion:

A novel *Acinetobacter sp.* PD 12 was isolated from feather waste dumping site from Chembur region, Mumbai, Maharashtra. The organism efficiently degraded feather keratin during submerged cultivation in medium containing whole feathers as the only source of carbon, nitrogen, sulphur and energy. Presence of extracellular protease was confirmed by zymogram analysis. The keratinolytic potential of this mesophilic strain could be potentially employed in the biodegradation of keratinous waste generated by commercial poultry processing industry. Microbial / enzymatic hydrolysis of such waste will not only reduce waste disposal problem, but will also help in generation of value added products such as protein hydrolysates for utilization as animal feeds or fertilizers.

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