INVESTIGATION OF KERATINASE ACTIVITY BY THERMO-ALKANOPHILIC *Nocardiopsis sp*. SD6 ISOLATED FROM FEATHER WASTE SOIL

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Abstract

The aim of the study was to isolate keratinolytic actinobacteria from feather waste soil, poultry farm soil in Tiruchirappalli and Nammakkal District, Tamil Nadu, India and their keratinase activity were investigated by feather degradation. Totally 91 isolates were obtained from feather waste soil and poultry waste soil samples. Among the 91 isolates, 32 positive isolates were selected after grown on modified starch casein agar (SCA) medium with additional casein. All 32 proteolytic isolates were subjected for casein hydrolysis and four significant isolates were selected based on their growth on basal liquid medium containing chicken feather. The isolate SD6 was tentatively identified one of the best among 4 isolates. The isolate SD6 was characterized by morphological, biochemical and molecular property inducing 16s rRNA analysis based and has been identified as Nocardiopsis sp. SD6 (JF907189). The isolate was grown on modified starch casein broth (SCB). Supernatant from centrifuged culture was examined for protease and keratinase activity. The optimum temperature and pH for enzyme activity for both proteolytic and keratinolytic was found at 50°C, pH 8 respectively. Extracellular keratinase enzyme was separated from the culture supernatant by centrifugation and precipitated with ammonium sulphate (80% saturation) and followed by dialysis using dialysis membrane 150. Native PAGE was performed with the enzyme sample and visible bands were observed after de-staining. Zymogram gel electrophoresis was employed to identify the keratinase enzymes band and three bands in the gel were found with positive keratinase activity.

Keywords: Feather Waste, Keratinase, *Nocardiopsis* sp., Zymogram.

1. Introduction

Keratins (KRTs) are the most abundant proteins in mammalian epithelial cells and the major components of skin, nail, hair, horn, feather, and wool. Two types of KRTs, α-KRTs and β-KRTs, consist of tightly packed protein chains in α -helices and β -sheets, respectively. Keratin filament structures are stabilized by their high degree of cross-linking of disulfide bonds, hydrophobic interactions, and hydrogen bonds (Parry and North, 1998). Feather waste, generated in large quantities as a byproduct of commercial poultry processing, is nearly pure keratin protein (Subhasish and Dhanasekaran, 2010). Due to their extremely rigid structures, KRTs are insoluble and hard to degrade. However, certain microorganisms synthesize keratinase that are able to degrade keratins. Keratinolytic activity has been reported for different bacterial and fungal genera (Mona, 2007). These enzymes enable keratinolytic bacteria and fungi to degrade waste keratin in nature (Lin *et al*., 1992).

Keratinase have several potential applications, such as in detergent formulations for eliminating horny epithelial cells adhered to textile fibers ecologically friendly leather processing waste chicken feather degradation (Parry and North, 1998), nutritional improvement of waste feather for livestock feed, production of protein hydrolysis's from keratinous waste materials (Kida *et al*., 1995), leather tanning and waste water treatment (Mitsuiki *et al*., 2004). It is also true that knowledge of the mechanism of keratin hydrolysis is very limited.

In this present study, we reported the keratinolytic nature of actinobacteria isolated from feather waste soil. Morphological, biochemical and molecular characterization of efficient keratinolytic Nocardiopsis sp. was described. Partial purification and characterization of keratinolytic enzyme was also estimated.

2. Materials and methods

2.1. Isolation of actinobacteria

The soil samples were collected from different feather waste dumping locations in Tiruchirappalli and poultry farms in Nammakkal, Tamil Nadu, India. Bennett's agar was used for the isolation of Actinobacteria by the method of Radhika *et al.,* 2008. One gram of soil sample was suspended in 9 ml of sterile distilled water and serially diluted and plated on Bennett's agar.

2.2. Primary and Secondary screening of keratinolytic actinobacteria

Milk agar medium was used for the primary screening of keratinolytic property for the isolated actinobacteria (Riffel and Brandelli, 2006). Skim Milk Powder was mixed separately in the medium prior to pouring the medium into petridishes to avoid the milk protein (casein) coagulation. The efficient keratinolytic isolates obtained through primary screening were subjected to secondary screening in Modified basal liquid medium supplemented with raw chicken feather, $MgSO_4$, $7H_2O$ 0.2 g/l; K_2HPO_4 0.3 g/l; KH_2PO_4 0.4 g/l; $CaCl_2$ 0.22 g/l and Yeast extract 0.1 g/l (Mona, 2008).

2.3. Characterization of Keratinolytic Isolate

The morphological and biochemical characterization of the keratinolytic isolate were carried out as described in International Streptomyces project (ISP). The morphology of the spore-bearing hyphae with the entire spore chain, substrate and aerial mycelium of the strain was examined under light microscope as well as by scanning electron microscope (Hitachi, S 3400 N). The optimal nutritional and cultural conditions was determined to identify the suitable media for growth, the isolate was inoculated in different culture media (SCA, ISP #1, ISP #2, ISP #3, ISP #4, ISP #5, ISP #6). Different nutritional amendments (carbon, nitrogen, starch, casein hydrolysis, nitrate reduction, urea utilization, NaCl concentration and other necessary biochemical parameters) and culturing conditions (incubation temperature and pH) were optimized for the maximal growth of the efficient keratinolytic isolates. The isolate was compared and identified according to Bergey's Manual of Determinative Bacteriology.

2.4. Isolation of chromosomal DNA

Culture broth (1.5 ml) was centrifuged at 8,000 rpm for 5 min at room temperature and the supernatant was discarded. The process was repeated to get enough cells mass. The genomic DNA was isolated following the modified method of Pospiech and Neumann, (1995). DNA concentration was evaluated using 0.8% (w/v) agarose gel electrophoresis stained with ethidium bromide and visualized using an Image Analyzer Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA, USA). Lambda DNA was used as control.

2.5. PCR amplification of the 16S rDNA

Genomic DNA was amplified with slight modification in PCR conditions as described previously (Stach *et al*., 2003). Actinobacteria specific forward [27F 5'-AGAGTTTGATCMTGGCTCAG-3'] and reverse [765R 5'-CTGTTTGCTCCCCACGCTTTC-3'] primers were used for the amplification of genomic DNA. Sequencing was carried out at the Xcelris Laboratories, Ahmadabad, India. The 16S rDNA sequence obtained was searched through GenBank database by using Blast algorithm to identify the closest matches. Sequence was aligned with representative actinobacterial 16S rDNA sequences and a phylogenetic tree was constructed using the CLUSTALX and Molecular Evolutionary Genetics Analysis (MEGA) software version 4.1.

2.6. Enzyme Assay

2.6.1. Proteolytic activity

The proteolytic activity was determined according to the modified method described by Mohamedin (1999). To 1.5 ml of 0.5% casein in 0.2 M borate buffer (pH 8) 0.5 ml of culture supernatant was added. The mixture was incubated at 50°C for 30 min. The enzyme reaction was stopped by the addition of 3 ml of 5% TCA. A control was run in an identical manner except that enzyme solution was added after the addition of TCA. After incubation, the reaction mixtures were kept at room temperature for 30 min and then filtered through Whatman No. 1 filter paper. The absorbance of TCA soluble fraction was read at 280 nm in a UV-visible spectrophotometer. One unit of proteolytic activity was determined as the amount of enzyme required to liberate 1 µg of tyrosine under specified conditions.

2.6.2. Keratinolytic activity

Keratinolytic activity was assayed by the modified method of Mohamedin, 1999. To 20 mg of feather barbs, cut with scissors into fine pieces, the following solutions were added i.e. 2 ml 0.2 M borate buffer (pH 8), 0.5 ml 0.01 M $MgCl_2$, 0.5 ml of culture supernatant and 3.5 ml of water. The mixture was incubated at 50^0C for 3h. At the end of incubation, the mixture was filtered through Whatman No.1 filter paper. Control was prepared in an identical manner except that enzyme solution was added after the filtration. One unit of keratinolytic activity was determined as the amount of enzyme that liberates 1µg of tyrosine under the above mentioned conditions.

2.6.3. Determination of optimum pH and temperature for enzyme activity

The optimum pH and temperature were determined by the similar method of determination of proteolytic activity. The pH and temperature stability were estimated by pH 4 – 11 and temperature 30°-70°C. The enzyme activity was quantified under standard assay conditions.

2.7. Ammonium sulphate fractionation

The culture supernatant was obtained by centrifuging the culture broth at 10000 rpm for 10 min at room temperature. The protein in the supernatant was precipitated by the addition of solid ammonium sulphate (60-80% saturation). The precipitate was collected by centrifugation at 12000 rpm for 10 min. The precipitate was dissolved in 100 mM Tris buffer (pH 8) and dialyzed using Dialysis Membrane 150 against the same buffer with two changes of 10h interval.

2.8. Protein Assay

The protein content of enzyme preparation was determined by using the modified method of Bradford, (1976).

2.9. Gel Electrophoresis

Non-denaturing polyacrylamide gel electrophoresis was carried out according to the method of Radhika *et al.,* 2008, using 12% cross-linked polyacrylamide gel.

2.10. Zymography

Zymogram analysis was based on a previously described procedure (Bressollier *et al.,* 1999: Ana Paula *et al.,* 2010), with modifications. Proteolytic enzyme was electrophoresed at room temperature on 12% non-denaturing polyacrylamide gel. After electrophoresis, the gels were washed with Tris–HCl buffer (20 mM, pH 8.0) containing 2.5% (v/v) Triton X-100 for 10 min. Casein (2%, wt/vol) in 50 mM Tris-HCl buffer (pH 8.5) was then poured onto the gel slab containing keratinolytic enzyme. After 3h of incubation at 40°C, the gel was stained with Coomassie brilliant blue R-250 and then destained. Protease bands appeared as clear zones on a blue background.

3. Results

3.1. Isolation of actinobacteria

Totally 91 actinobacterial isolates were isolated from feather waste soil and poultry waste soil samples from different locations in Tiruchirappalli (10.8050°N 78.6856°E) and four poultry farms in Nammakkal (11.23°N 78.17°E), Tamil Nadu, India (Table 1). All isolates were marked as SD and alphabetic number were given respectively from 1 - 91.

Table 1. Population of Actinobacteria isolated from Feather Waste Soil

Sl.No	Sampling Site	Nature of the Collection locations	Total Actinobacterial Population CFUx10^6/g
1.	Mathur, Tiruchirappalli Sample Site 1	Feather Dumped Soil	16 (17.58%)
2.	Mathur, Tiruchirappalli Sample Site 2	Feather Dumped Soil	12 (13.20%)
3.	Mathur, Tiruchirappalli	Human Hair Dumped Soil	9 (9.89%)
4.	Vayaloor, Tiruchirappalli	Feather dumped soil	14 (15.38%)
5.	Balasubramani Poultry Farm, Thopur, Seethapatty Road, Mohanur, Namakkal (Egg Production)	Sample 1: Soil, Inside the farm, Working Condition Sample 2: Dried Fecal, Inside the farm, Not in Working Condition	8 (8.79%) -
6.	Siva Shakti Poultry Farm, Thopur, Seethapatty Road, Mohanur, Namakkal (Egg Production)	Sample 1: Sick Chickens Cage's soil sample Sample2: Soil + Fecal, Inside the Farm, Not in Working Condition	3 (3.29%) 7 (7.69%)
7.	Chinaswamy Poultry Farm, Namakkal Veterinary College Mohanur Road Namakkal - 2 (Egg Production)	Sample 1: Sick Chickens Cage's soil sample, Not in Working Condition Sample 2: Soil Sample, elevated farm (30-40 ft from the ground level)	- 13 (14.28%)
8.	Vardhraj Poultry Farm Namakkal Veterinary College Mohanur Road Namakkal – 2 (Egg Production)	Sample 1: Dried Fecal + soil Sample 2: Dried Soil, Not in Working Condition	3 (3.29%) 6 (6.59%)

3.2. Primary and Secondary screening of keratinolytic actinobacteria

The isolate SD6 has shown significant degradation of casein (Figure 1) in primary screening among the 91 total actinobacterial population isolated from feather waste soil samples and also observed with potential feather degradation capability (Figure 2) through secondary screening.

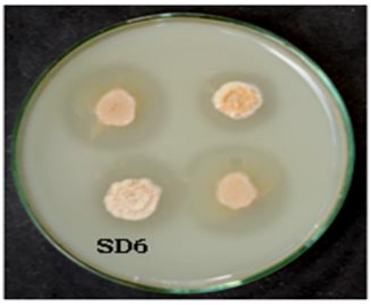


Figure 1. Primary screening of isolate SD6 in milk agar medium.

Figure 2. Secondary screening of isolate SD6 in modified basal liquid medium.

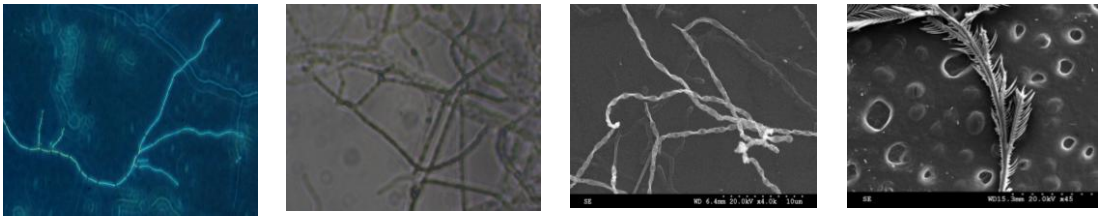
3.3. Characterization of Keratinolytic Isolate SD6

The morphological (Figure 3; 4 and Table 2) and biochemical characteristics (Table 3) of the keratinolytic isolate SD6 were tentatively suggested the distinct characters of *Nocardiopsis* sp.

Table 2. Morphological characteristics of isolate SD6

Morphological Characterization	Isolate SD6
Colour of Arial Mycelium	White
Colour of Substrate Mycelium	Yellowish
Sporephore	Non Spiral
Spore Shape	Short Rod with Rounded End
Spore Surface	Smooth
Pigment Production	Nil

Figure 3. Isolate SD6 on ISP5 medium



(a) (b) (c) (d)

Figure 4. Morphological characterization of isolate SD6: (a) Dark field Microscopic view, (b) Light Microscopic view, (c) SEM view, (d) SEM view of degraded feather by SD6

Table 3. Biochemical Characteristics of Keratinolytic Isolate SD6

Medium	Isolate SD6
ISP1	
Growth:	**+++**
Aerial Mycelium:	**White**
Substrate Mycelium:	**White**
Pigmentation:	**Nil**
ISP2	
Growth:	**++++**
Aerial Mycelium:	**White**
Substrate Mycelium:	**Yellowish**
Pigmentation:	**Nil**
ISP3	
Growth:	**+++++**
Aerial Mycelium:	**White**
Substrate Mycelium:	**Gray**
Pigmentation:	**Nil**
ISP4	
Growth:	**+++++**
Aerial Mycelium:	**White**
Substrate Mycelium:	**Gray**
Pigmentation:	**Nil**
ISP5	
Growth:	**+++**
Aerial Mycelium:	**White**
Substrate Mycelium:	**White**
Pigmentation:	**Nil**
ISP6	
Growth:	**++**
Aerial Mycelium:	**White**
Substrate Mycelium:	**Gray**
Pigmentation:	**Nil**

Test	Isolate SD6
Indole	**-**
MR	**-**
VP	**-**
Citrate Utilization	**+**
TSI	**K/K**
Gas Production	**-**
H_2S Production	**-/+**
Catalase	**++**
Oxidase	**+**
Starch Hydrolysis	**++**
Casein Hydrolysis	**++**
Nitrate Reduction	**-**
Urea Utilization	**+**
NaCl Tolerance	
0%	**Moderate**
2.5%	**Luxuriant**
5%	**Moderate**
7.5%	**Moderate**
pH for Growth (Optimum)	**9**
Temperature (Optimum)	**45 – 50^{0}C**
Best Carbon Source	**Sucrose**
Best Nitrogen Source	**Asparagine and Serine**

3.4. Phylogenetic Analysis

The partial sequencing of 16S rRNA gene of the isolate SD6 on both directions yielded 16S rDNA nucleotide sequence with 1,000 base pairs. Sequence of the isolate was deposited in the GenBank (NCBI, USA) with the accession number JF907189. A neighbor-joining tree with bootstrap value of the sequences showed that the isolate occupies a distinct Phylogenetic position within the radiation including representatives of the *Nocardiopsis* family (Figure 5).

3.5. Proteolytic and keratinolytic activity for isolate SD6

The proteolytic activity with casein substrate was determined for *Nocardiopsis* sp. SD6. The degradation of intact feathers by isolate SD6 was evaluated in terms of keratinolytic activity was found to be significant (Table 4).

3.6. Effect of pH and temperature for enzyme activity

The effects of pH and temperature on keratinolytic activity was investigated. Alkaline pH and higher temperature were found to be suitable for keratinolytic activity (Figure 6).

3.7. Non denaturing polyacrylamide gel electrophoresis analysis

The ammonium sulphate precipitated (80%) supernatant was dialyzed and analyzed for protein content (Table 4). Non denaturing polyacrylamide gel electrophoresis (ND-PAGE) showed the protein profile of the dialyzed protein obtained from *Nocardiopsis* sp. SD6 (Figure 7).

3.8. *Zymography*

Zymogram study against casein substrate observed with active enzyme bands of *Nocardiopsis* sp. SD6 on the ND-PAGE (Figure 8)

Table 4. Proteolytic, Keratinolytic activity and protein content of *Nocardiposis* sp. SD6

Test	*Nocardiopsis* sp. SD6
Proteolytic activity	25 unit/ml
Keratinolytic activity	18 unit/ml
Protein content	20 µg/µl

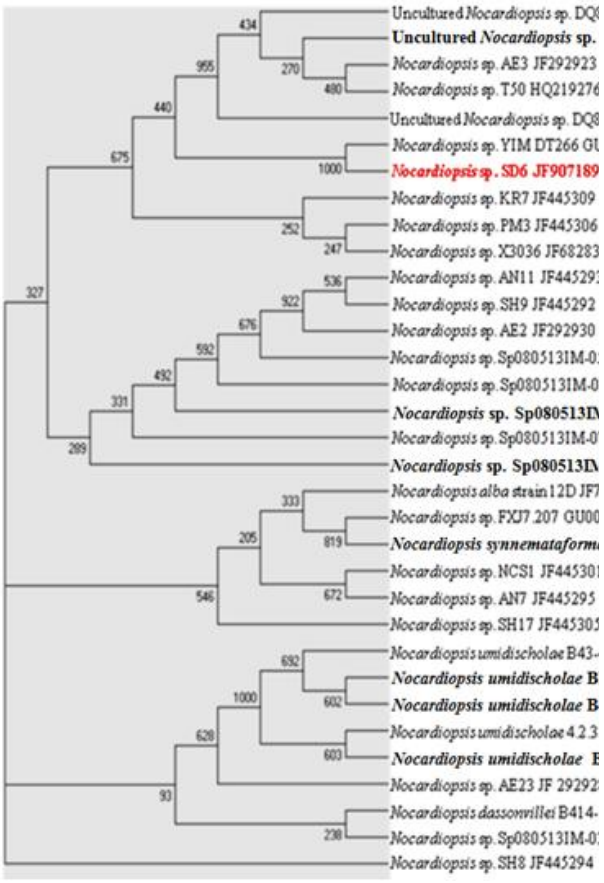


Figure 5. The phylogram showing the position of the isolate SD6 with other *Nocardiopsis* sp. based on 16S rDNA sequence

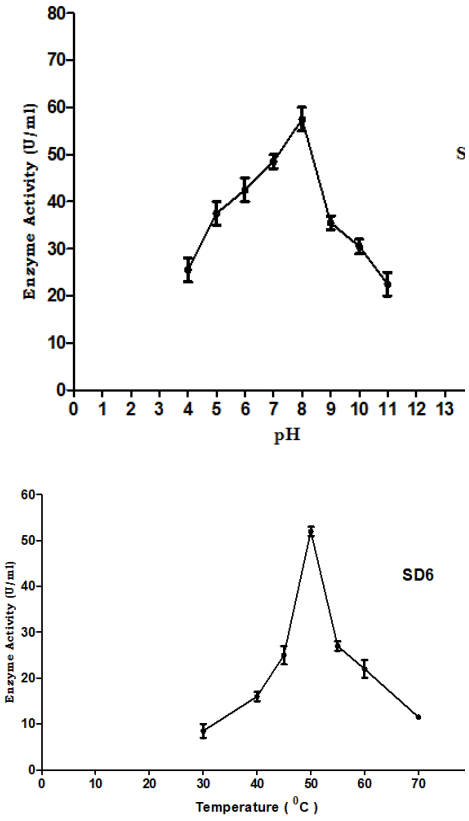


Figure 6. The optimum pH and temperature for proteolytic and keratinolytic activity of isolate SD6.

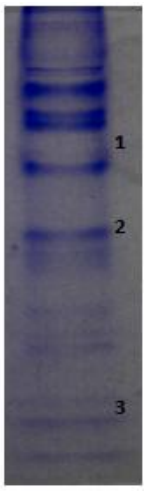


Figure 7. ND-PAGE Figure 8. Zymogram

4. Discussion

Totally 91 isolates were isolated from different feather waste soil samples. All isolates were initially screened for proteolytic activity. Among them 15 isolates showed maximum protease activity on milk agar medium and they were further investigated for keratinase activity in terms of degrading raw feather. The isolate SD6 was found to have significant keratinase activity. The morphological and biochemical characteristics revealed that the isolate was Gram positive, non pigmented and spore forming with whitish aerial mycelium and yellowish substrate mycelium. SEM analysis of isolate SD6 showed the long chain of spores with short rod and rounded ends. The isolate SD6 was found to grow optimum on ISP 3 and ISP 4 medium.

On starch and casein hydrolysis, isolate observed with hydrolysis zone on the respective medium. The isolate was found positive for urea utilization, nitrate reduction, citrate utilization, H_2S production and efficient to tolerate beyond 2.5% of NaCl. Among 10 different sugars and amino acids, sucrose was found to be utilized as suitable carbon and asparagine and serine were found as suitable nitrogen source respectively. pH 9 and 50^0C were supporting the optimal condition for the growth of isolate SD6. The phylogenetic relationship revealed that isolate SD6 was strongly related with other *Nocardiopsis* sp., thus isolate SD6 was justifiably identified as *Nocardiopsis* sp. SD6 and the 16S rDNA nucleotide sequence of the same isolate was deposited in NCBI database with the accession number JF907189. The degradation of intact feather using supernatant of *Nocardiopsis* sp. SD6 was achieved in 96-120 hours of incubation at 42-50^0C. SEM analysis of degraded feather showed extensive degradation of keratin. The enzymatic assays suggested the significant capability of extracellular keratinolytic enzyme released by *Nocardiopsis* sp. SD6. The characteristics of *Nocardiopsis* sp. SD6 were found to be similar with the findings of Wael and Goodfellow, (2008) and Vimal *et al.*, 2009. Like the general nature of keratinase enzyme, *Nocardiopsis* sp. SD6 produced keratinolytic enzyme also showed the stability at alkaline pH and high temperature. The currently obtained findings for degradation of chicken feathers at alkaline pH and high temperature were also observed with the other actinobacteria, especially *Streptomyces* (Sangali and Brandelli, 2000; Riffel *et al*., 2003; De Azeredo *et al*., 2006). Saturated ammonium sulphate (80%) which precipitated extracellular enzyme was dialyzed to

remove the salts. The complete removal of salt from precipitated enzyme was confirmed by Nessler's reagents. Protein content in the salt free precipitated enzyme solution suggested that *Nocardiopsis* sp. SD6 is capable to produce a good amount of extra cellular keratinolytic enzyme upon the favorable conditions provided. ND-PAGE detect the protein profile of the keratinolytic enzyme obtained from isolate SD6 and Zymogram studies indicated the responsible bands with keratinolytic activity. Significant reports on keratinase from *S. pactum* DSM 40530, *Streptomyces* sp. SK1-02, *Scopulariopris brevicaulis*, *B. licheniformis* and *K. rosea* have been observed by Bressollier *et al*., (1999); Bockle *et al*., (1995); Letourneau *et al*., (1998); Bernal *et al*., (2006), Rozs *et al.,* (2001); Brandelli and Riffel, (2005); Riffel *et al.,* (2003); Radhika *et al*., (2008), but very minimum work has been done with *Nocardiopsis* sp. on keratinase aspect. Only Shinji *et al.,* (2004) has done similar sort of work with *Nocardiopsis* sp. TOA-1 on NAPase gene and considering the fact of minimum data on keratinase activity from *Nocardiopsis* sp., findings of present study were praiseworthy.

5. Conclusions

The present study revealed the novel role of *Nocardiopsis* sp. in chicken feather waste degradation. Feather is a rich source of keratin protein and degraded keratin can play a remarkable source of amino acids for animal feed stock, manure etc., formulations. Almost from last two decades it is being tried to convert waste feather into usable byproduct. Degradation of feather by means of physical and chemical treatment is not capable to produce the usable byproduct. Actinobacterial degradation of feather can be a good choice to derive optimum usable byproduct from feather waste using keratinase enzyme producing *Nocardiopsis* sp. SD6.

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