IJBPAS, February, 2023, 12(2): 572-584

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

ISOLATION, IDENTIFICATION, AND CHARACTERIZATION OF KERATINOLYTIC BACTERIA FROM POULTRY FEATHER AND POULTRY FEATHER DUMPING SOIL

P.KAVITHA[1] AND N. JAYANTHI[*2]

1: Research Scholar, Marudupandiyar College, Thanjavur

2: Assistant Professor, Mannar Thirumalai Naicker College, Madurai

***Corresponding Author: Hiral S. Popaniya: E Mail: hpopaniya@gmail.com**

Received 29th March 2022; Revised 28th April 2022; Accepted 17th July 2022; Available online 1st Feb. 2023

https://doi.org/10.31032/IJBPAS/2023/12.2.6822

ABSTRACT

Environmental pollution is an issue of global concern and feather waste which is a by-product of the growing poultry industry is an example of a pollutant not easily degraded by common proteolytic enzymes. However, feathers when acted upon by keratinolytic microbes are degraded to smaller peptides and amino acids which can be used as dietary supplements in animal feed. Keratin is an insoluble protein macromolecule with very high stability and a low degradation rate. Keratin is mainly present in hair, feathers, nails, wool, and horns. The present study deals with the isolation, identification, and characterization of keratinolytic bacteria from chicken feathers. One type of poultry feather and One Type of poultry feather dumping soil were collected from Melmangalam and Vadugapatti in Tamilnadu, South India. Two types of feather degrading keratinolytic *Bacillus sp* ecies were isolated and identified using various microbiological and biochemical procedures. They were screened by their growth and feather degrading ability on feather meal agar, and they were named *Bacillus sp* 1 and 2. The proteolytic activities of the isolates were screened on casein agar. Secondary screening of the isolates was determined by keratinase activity. The isolates were confirmed as *Bacillus cereus and Bacillus subtilis* by 16S rDNA sequencing.

Keywords: Chicken feather, Keratinolytic bacteria, *Bacillus sp* , proteolytic activity

INTRODUCTION

Increasing commercial poultry processing, feather waste has been generated in large quantities as a by-product **[1]**. Poultry waste materials cause a harsh effect on the environment. Worldwide around 8.5 billion tons of chicken feathers are produced annually as a waste from the production unit of chicken meat. In India, about 350 million tons of chicken waste has been produced annually **[2]**. Feathers represent 5-7% of the total weight of mature chickens **[3]**. Chicken feathers contain nutrients of approximately 91% protein (Keratin), 1% lipids, and 8% water **[4]**. Feathers are rich in pure keratin protein **[5]**. Keratin is an insoluble structural protein in epithelial cells of vertebrates and represents the major constituents of skin, hair, feather, wool, hooves, and scales **[6]**. These specific proteins which belong to the sclera protein groups are compounds that are resistant to physical, chemical, and biological actions. Mechanical stability and high resistance to proteolytic degradation or keratin are due to the presence of disulfide bonds, hydrogen bonds, salt linkages, and cross-linkages **[7]**.

Feathers are also converted to feather meal with usage as animal feed, organic fertilizers, and feed supplements, as it is made up of 90% protein and is rich in hydrophobic amino acids like cysteine, arginine, and threonine **[8]**. Keratin waste can be used as a good source of alternative protein feedstuff. A number of methods are used for the hydrolysis of chicken feathers. Chicken feather keratin when treated with lime (Calcium hydroxide) to get a liquid product rich in amino acids and polypeptides, can be used as an animal feed supplement. Industrially feather meal is produced by either physical (steam) or chemical treatment methods **[9]** or buried in landfills and burning. Physical and chemical methods of feather decomposition can cause contamination of air, soil, and water **[10]**, and lead to cause environmental pollution **[11]**. Therefore, an urgent requirement is needed to convert these waste materials to potentially useful products. At present, the poultry industry manages waste (feather carcass, hair, and manure) through several disposal methods. One such method is called composting. It is one of the more economical and environmentally safe methods of recycling the feather **[12]**. But, feather keratin is not easily degraded by normal proteolytic enzymes. A number of technologists for the bioconversion of keratinous waste into value-added products **[13]** are available today. Biodegradation of intensively farmed animal waste is now viewed as an alternative avenue for creating a viable end product with visible benefits to the primary producers in the environment and a more

used/efficient economic management strategy **[14]**. Microbial conversion is the best and pollution-free method for removal of such waste materials into useful products. Microorganisms are widely distributed in nature and have a major role in the degradation of organic matters. Especially, soil microorganisms have the potential for utilizing feather keratin as a carbon and energy source. Microbial keratinolytic enzymes may be used to enhance the digestibility of feather keratin **[15]** and have many applications in feed, fertilizer, detergent, leather, and pharmaceutical industries **[16]**. Certain microorganisms including bacteria, fungi, and actinomycetes readily decompose keratin-based materials by their extracellular enzyme named keratinase **[17]**. Keratinolytic activity has been reported for species of *Bacillussubtilis, B. cereus, B.amyloliquefaciens, B. megaterium, and Bacillus sp* . **[18]** Streptomyces **[19]**, and actinomycetes **[20]**. Keratins are insoluble macromolecules that comprise a tight packing of supercoiled long polypeptide chains with a molecular weight of approximately 10 k Da. The high degree of cross-linked cystine disulfide bonds between contiguous chains in keratinous material imparts high stability and resistance degradation (*Bacillus subtilis* and *Bacillus cereus*). Hence, a keratinous material is a tough, fibrous matrix being mechanically firm, chemically unreactive, water-insoluble, and protease-resistant (*Bacillus subtilis* and *Bacillus cereus*). Such a molecular structure makes feathers poorly degradable under aerobic digestion condition.

MATERIALS AND METHODS

Sample Collection

One type of poultry feathers such as raw feather from the poultry farm and one type of poultry feather dumping soils were collected from a poultry farm located at Melmangalam and Suguna Chicken Shop at Vadugapatti, Theni District, Tamil Nadu, South India. The collected samples were transferred to sterile plastic bags and transported to the microbiological laboratory and stored at 4ºC for further study.

Isolation and characterization of bacteria

Isolation of keratinolytic bacteria was performed with feather dumping soil and feather. One gram of feather-dumped soil samples was transferred to 99 ml of sterilized distilled water and mixed properly. Serial dilutions were made up to 10^{-9} dilutions. The samples were taken from 10^{-5} and $10^{-6}$for the isolation of keratinolytic bacteria using nutrient agar medium and after inoculation nutrient agar plates were incubated at 37^{0}C for 24-48 hours. One gram of raw feather was trimmed into bits and added to the nutrient

broth for the isolation of keratinolytic bacteria. The broth was incubated at 37^0C for 24hrs. After incubation, the colonies and inoculums were subjected to various microbiological and biochemical tests and 16 sRNA sequencing. Further, these colonies were cultured on NYSM medium and Skim Milk Agar medium for screening of keratinolytic bacteria.

Determination of Feather Degradation

The feather degradation was determined according to the method described by **[21]**. The 2 bacterial isolates from the raw feather and feather dumping soil sample (FDS-1) were inoculated in feather meal broth with 1% feather as the sole sources of carbon and nitrogen and incubated on a rotary shaker for one week. After one week the residual remained was determined gravimetrically by filtering the culture broth and taking the weight of filter paper before and after filtration.

Percent reduction of feathers was calculated from the difference in the initial weight and weight obtained after one week of incubation.

$$\text{Percent Weight loss} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

Assay for Keratinase Activity:

Keratinase activity was assayed according to the method of **[21]**. Each culture filtrate was centrifuged at 5000 rpm for 30 min. 20mg feather meal powder, 11.4 Tris HCl buffer, 0.6 ml supernant of culture filtrate were taken; control was kept where 0.6 ml cultures supernatant was replaced with distilled water. The tubes were incubated at 30°C for 1 hour then chilled in ice water for 10 minutes filtered and OD was calculated by using following formula:

Enzyme units per ml = Optical density * 12 * Dilution rate / 0.01 * T

Where T = Incubation time; 12 = Total volume used.

Protein Determination:

The protein content of samples was analyzed using the lowery methods with bovine serum albumin as standard protein **[22]**.

RESULTS

Isolation of keratinolytic Bacteria from Feather Dumping Soil and Chicken feather

Feather Dumping Soil

Isolation of bacteria was performed by serial dilution and plating method on nutrient agar medium. After incubation, the colonies were observed, in round shape, 2 mm in size, creamy in color, rapid growth, and the opaque is formed.

Chicken feather

Raw feather was cut into small pieces and put into the nutrient broth. After incubation, the organisms are grown in nutrient broth. It was utilizing the nutrients and feather organisms are grown, the turbidity is formed. The bacterial isolates are streaked in nutrient agar plates. After the 37°C incubation, the colonies are grown only in streaking areas. Morphological characterizations of isolates were analyzed in **Table 1**. The biochemical characterization resulted in **Table 2**.

Biochemical Characterization:

The bacterial isolates were characterized biochemically by the Indole test, Methyl red test, Vogus Proskauer's test, Citrate Utilization test, Urease test, Nitrogen Reduction test, and Trible Sugar Iron agar test **[23]**.

Screening of keratinolytic bacteria

Primary Screening of Keratinolytic Bacteria:

The isolates were streaked in Skim Milk Agar Plates after 24 hrs incubation at 37^0C.The zone formed around the colonies due to the production of protease enzyme.NYSM medium (Nutrient Yeast Salt medium) was prepared and the isolates were streaked on the medium after 24-hour incubation at 37°C. The selected species were grown on the medium.

Secondary screening of Keratinolytic Bacteria:

The primary screening positive organisms are inoculated in feather meal broth. Cultures were grown at 37^0C at 120 rpm for 96 hrs. Keratinolytic strain that completely breaks down feathers in the medium. The feather was surface sterilized. After sterilization, the feather was used for Keratinase production.

Identification of keratinolytic Bacteria:

The isolates are streaked in Feather meal agar plate. Feather meal is the only source of Carbon and Nitrogen. The plates are incubated at 37^0c at 48-72. After incubation, the clear zone occurs. It indicates Keratinolytic bacteria utilize the feather and grow. So, a clear zone are formed.

Feather meal powder preparation:

The feather meal powder uses in various test of keratinolytic bacteria, it used in Keratinase activity determination and feather meal broth, and feather meal agar preparation.

Production of Keratinase enzyme and feather degradation by bacterial isolates:

The keratinase enzyme is produced in feather meal broth and inoculation of samples, using a rotary shaker at 120 hrs.

Incubation. After incubation Keratinolytic strain completely broken down feathers in the medium. Its known feather degradation is done by bacteria and the production of keratinase enzyme.

Extraction of enzyme:

Whatman No. 1 filter paper is used to remove un degraded residues. The filtrate was then subjected to centrifuge at 10,000 rpm for 10 mins to remove the bacterial residues. After centrifugation keratinase activity was determined in the supernatant. The supernatant is known as an enzyme.

Determination of Feather Degradation:

After one week of incubation the percent weight loss calculated is shown.

The determination of feather degradation by the bacterial isolates viz Raw Feather and FDS-1. The results infer with the maximum degradation with FDS-1 (90.8%) followed by Raw Feather (87.6%) respectively. The minimum degradation is with Raw Feather (87.6%). Hence the results show that the maximum degradation was determined in FDS-1 (90.8%). Similar results have been observed with *Pseudomonas microphilus* (70%) in 30 days and *Leuconostoc sp.* (31%) **[24] (Table 3)**.

Assay for Keratinase Activity:

After one week of incubation, the isolates were keratinase produced in different incubation periods. The od value was taken at 540 nm and od values were converted in unit/ ml.

The keratinase production of maximum production by *Bacillus sp* 2 (5.52 ± 0.04 unit/ml) at 72 hrs followed by *Bacillus sp* 1 (4.56± 0.03 unit/ml) 72 hrs. The minimum production by *Bacillus sp* 2 (1.32 ± 0.01 unit/ml) at 24 hrs followed by *Bacillus sp* 1 (1.56±0.01 unit/ml) at 24 hrs. Hence the results show that the maximum production of keratinase was *Bacillus sp* 2 (5.52 ± 0.04 unit/ml) at 72 hrs. Similar results have been observed with *Bacillus megaterium* (1.5%) concentration **[25]** and *Bacillus sp* (780 U/ml) at 48 hrs **[26] (Figure 2)**.

Protein Estimation:

The protein estimation was done by **[22]**. With serum albumin as a standard. All the solutions were mixed and after 30 mins OD value was noted in 540 nm **(Figure 3)**.

The Protein Estimation by *Bacillus sp* 1 and *Bacillus sp* 2 results with the maximum production by *Bacillus sp* 2 (1.44 ± 0.02 unit/ml) at 72 hours followed by *Bacillus sp* 1 (1.22 ± 0.01 unit/ml) at 72 hours. The minimum production by *Bacillus sp* 1 (0.53 ± 0.003 unit/ml) at 24 hours followed by *Bacillus sp* 2 (0.54 ± 0.003unit/ml) at 24 hours respectively. Hence the results show that the maximum production of keratinase was *Bacillus sp* 2 (1.44 ± 0.02 unit/ml) at 72 hours. The similar results were observed with

Pseudomonas microphilus (0.548 mg/ml) at 30 days **[24]** and *B.licheniformis*1274 & *B.subtilis* 1237 (4.5 mg/ml) at 7 days **[27]**.

Sequence Result:

The Sanger's Di Deoxy method of sequencing confirmed Rawther and Feather Dumping Soil sample 1 as:

Raw feather – *Bacillus cereus*

FDS – 1 - *Bacillus subtilis*

The bacterial isolate identified using biochemical characteristics are molecularly analyzed with BLAST n and CLUSTAL. The comparative analysis shows 99% similarity with *Bacillus subtilis* and *Bacillus cereus*

Table: 1 Morphological Characterization of Isolates

S. No.	Morphological characteristics	Samples	
		RF	FDS
1	Color	Creamy	Creamy
2	Size	2mm	2mm
3	Simple Staining	Rod Shape	Rod Shape
4	Gram Staining	Gram Positive	Gram Positive
5	Spore Staining	Spore Forming	Spore Forming
6	Motility Test	Motile	Motile

Note: RF – Raw Feather, FDS – 1– Feather Dumping Soil

Table 2: Biochemical Characterization of Isolates

S. No	Samples	Biochemical tests										Growthin specific media		Result
		C	O	CU	MR	VP	I	U	GH	NR	T S I	Skim milk agar	NYS M media	**Interferred Species**
1	RF	+	+	+	-	+	-	-	+	+	+	+	+	*Bacillus sp* 1
2	FDS-1	+	+	+	+	+	+	-	+	+	+	+	+	*Bacillus sp 2*

Note: RF – Raw Feather, FDS – 1 – Feather Dumping Soil

Note: C – Catalase test, O – Oxidase test, C U - Citrate Utilization test, MR – Methyl red test, VP- Voges Proskauer test, I – Indole test, U – Urease test, GH – Gelatin Hydrolysis test, NR - Nitrate reduction test, TSI – Trible Sugar Iron agar test.

Table 3: Determination of feather Degradation

S. No	Percent of weight loss	RF (g)	FDS-1 (g)
1	Initial Weight	1g	1g
2	Final weight	0.124g	0.092g
3	Initial weight – Finalweight / ×100 / Initial weight	87.60%	90.80%

Note: RF – Raw Feather , FDS-1 – Feather Dumping Soil (Sample) – 1

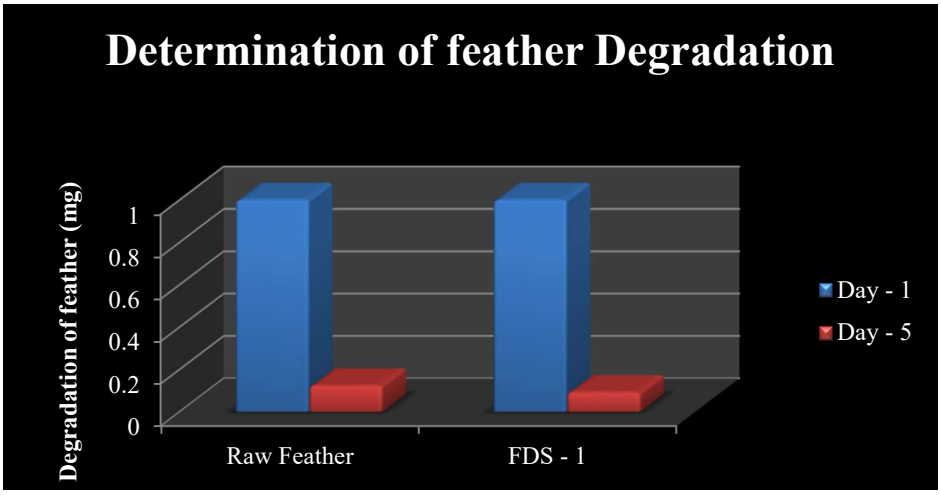

Figure 1: Determination of feather Degradation

Table 4: Keratinase production

S. No	Time duration (Hours)	Isolates			
		Bacillus sp 1 (unit/ml)		*Bacillus sp 2* (unit/ml)	
	X	Y	y Er±	Y	y Er±
1	24	1.56	0.01	1.32	0.01
2	48	3.36	0.02	3.48	0.02
3	72	4.56	0.03	5.52	0.04
4	96	3.6	0.02	3.36	0.02
5	120	2.04	0.02	1.92	0.01

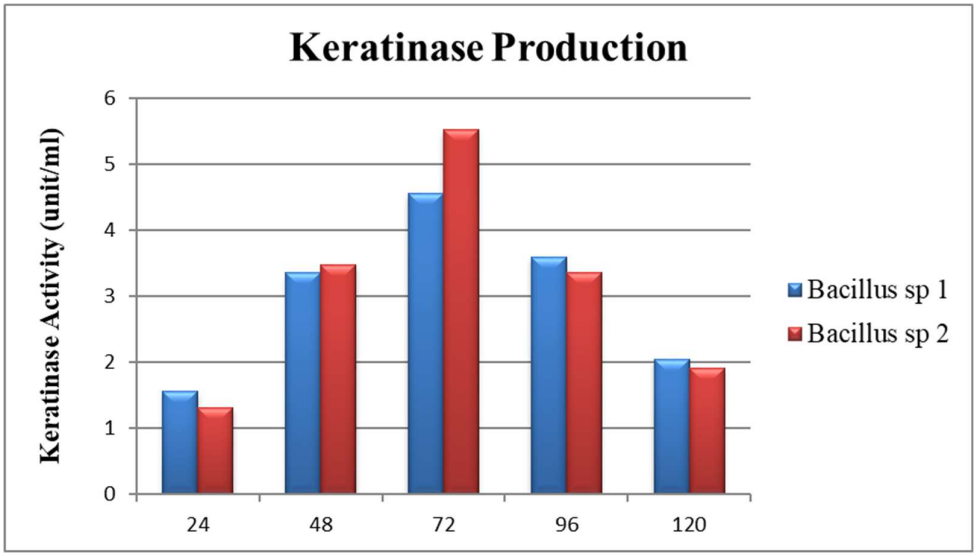

Figure 2: Keratinase Production

Table 5: Protein Estimation

S. No	Time duration (Hours)	Samples			
		Bacillus sp 1		*Bacillus sp 2*	
		(595 nm)		(595 nm)	
	X	Y	y Er±	Y	y Er±
1	24	0.53	0.003	0.54	0.003
2	48	0.81	0.004	0.88	0.004
3	72	1.22	0.01	1.44	0.02
4	96	1.09	0.01	1.32	0.01
5	120	0.91	0.004	0.72	0.003

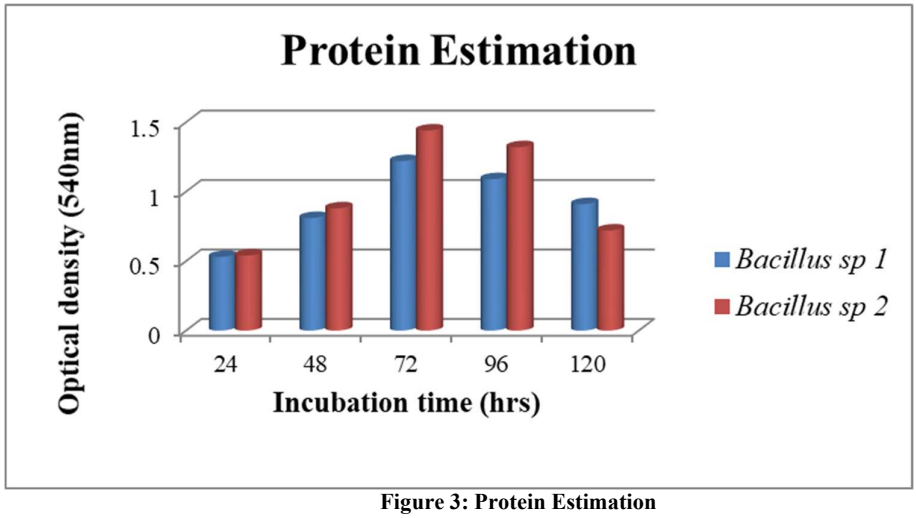

Figure 3: Protein Estimation

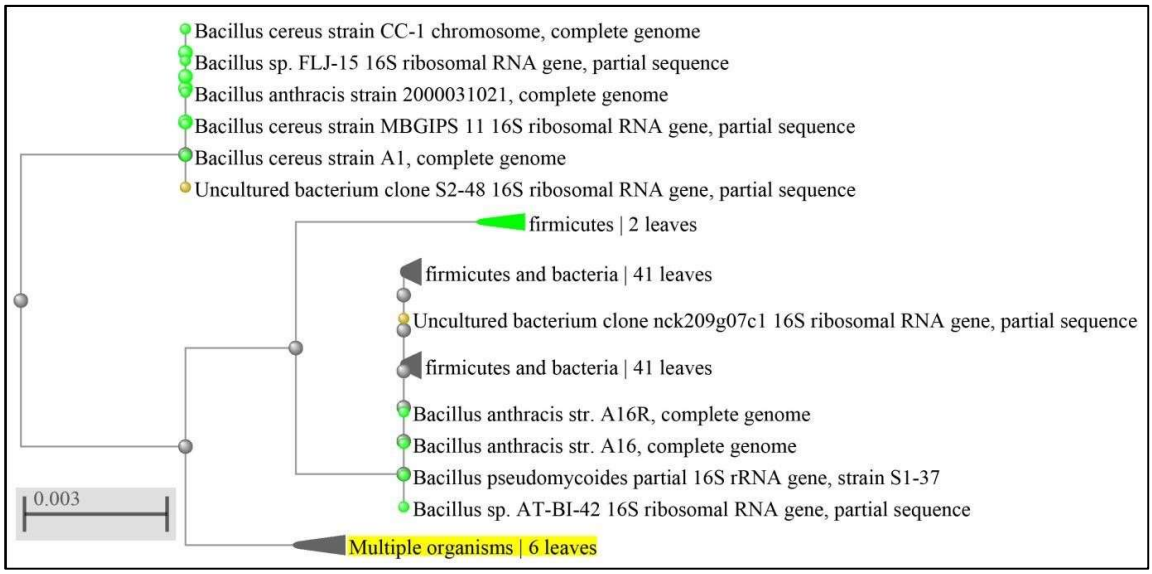

Figure 4: Phylogenetic tree of *Bacillus subtilis*

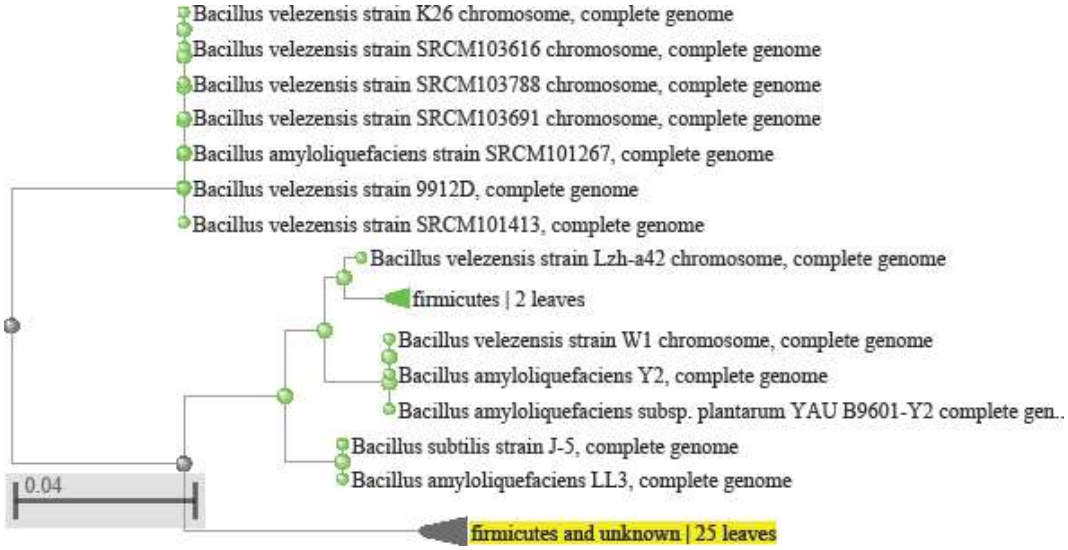

Figure 5: Phylogenetic tree of *Bacillus cereus*

CONCLUSION

In the present study, four bacterial cultures were isolated producing keratinase from habitats where keratin-containing substrates were disposed of in natural conditions. Bacteria were isolated by serial dilution method and plating method. The two bacterial isolates were characterized and identified based on colony morphology, growth characteristics, and Biochemical characteristics. All the isolates were performed in selective media growth. They were identified as belonging to genera *Bacillus species* 1 and 2. The isolate *Bacillus sp* 2 shows the highest feather degradation of (5.52 ± 0.04 unit/ml) at 72 hrs.

The ability of newly isolated bacteria to degrade feathers can be utilized for their potential biotechnological application in the processing of feather waste from the poultry industry. The evaluation of the biotechnological application of keratinase, however, requires a more detailed understanding of the factors that enable this enzyme for the complete degradation of the native keratinase substrate. Therefore additional research will need to be done for purification, Characterization of keratinase, studying the kinetics of enzymes, testing from various ranges of the substrate, the effect of inhibitors, and inducer on enzyme activity, submerged state fermentation for large scale production of keratinase. Further studies can be utilized for feather degradation, rather than individual cultures for enhanced keratinolytic activity. On the basis of data obtained in the present work, it can be concluded that *Bacillus sp* . Can be employed in the production of Keratinase. The degradation of feathers with

Keratinolytic bacteria is the best eco-friendly approach in poultry feather waste management.

An environmental threat is caused by poultry processing industries. Such industries are dumping their waste (chicken feathers) in the environment in large quantities. Landfilling, burning, production of natural gas and fertilizer are the primitive methods to remove the bulk feather waste.

REFERENCE

[1] Williams M, Ritchter S, Mackenzine M, ShihH (1990) Isolation, Identification and Characterization of feather degrading bacterium. Appl Enviro Microbiol. 56(6): 1509 –1515. Zaghloul TI, Embaby AM, Elmahdy AR. Key determinants affecting sheep wool biodegradation directed by a keratinase-producing *Bacillus subtilis* recombinant strain. Biodegradation.2011;22:111–128. doi: 10.1007/s10532- 010-9381-9.

[2] Sarita Agrahari, Wadhwa N (2010) Degradation of chicken feather a poultry waste product by Keratinolytic bacteria isolated from dumping site at Ghazipur poultry processing plant. International journal of poultry science. 9: 482-489

[3] Joshi S.G., Tejashwini M.M., Revati N., Sridevi R., Roma D. [2007]. Isolation, identification and characterazation of afeather degrading bacterium. International Journal of Poultry Science, No.6[9],. p. 689-693.

[4] Kannapan Saravanan, Bharathi Dhurai, 2012. Exploration on amino acid content and morphological structure in chicken feather fiber, JTATM 7:3.

[5] Moran ET, Gous RM, Harrison MD (1997) Effect of age on feather amino acid content in two broiler strain crosses and sexes. Journal of Applied Poultry Research 6:205-209.

[6] Kim B, Choe E, Hwang J, Suhartono M, Pyun Y (2002) Native-feather degradation by *Fervidobacterium islandicum* AW-1, a newly isolated keratinase-producing thermophilic anaerobe. Arch Microbiol 178:538-547.doi:10.1007/s00203-002-0489-0

[7] Thyagarajan, D., Barathi, M., Sakthivadivu, R., 2013. Scope of poultry waste utilization. IOSR J. Agric. Vet. Sci. 6, 29–35.

[8] Ekta T, Rani G (2012) Rapid conversion of chicken feather to feather meal using dimeric keratinase from *Bacillus licheniformis* ER-15. J Bio proceaa Biotech 2:4.

[9] Zheljazkov VD, Nielson NE. Effect of heavy metals on peppermint and cornmint Plani Soil. 1996; 178:59-66.

[10] Brutt, E.H. & Ichida, J.m. (1999) US Patent 5,877,000.

[11] Korkmaz H, Hur H, Diyncer S (2004) Characterization of alkaline keratinase

of *Bacillus licheniformis* strain HK-1 from poultry waste. Ann Microbiol 54:201–211

[12] Ichida JM, Krizova L, Lefevre CA, Keener HM, Elwell D, Burtt EH (2001) Bacterial inoculums enhances keratin degradation and biofilm formation in poultry compost. J Microbial Methods 47(2):199-208.

[13] Matsumata, T. (1996) In: Principle and practice of clinical mycology, (Eds, Kibbler, C.C., Mackenzie, D.L. & Odds F.C.) Wiley & sons Ltd, USA, pp. 103-129.

[14] W.K. Kim and P.H. Patterson (2000) Nutritional Value of Enzyme or Sodium Hydroxide – Treated Feathers from dead Hens.

[15] Onifade, A.A., Al-Sane, N.A., Al-musallam, A.A, Al-Zarban, S., 1998, "A review: potentials for biotechnological applications ofkeratin-degrading microorganisms and their enzymes for nutritional impovemnt of feathers and other keratins as livestock feed resources". Bioresources. Techno., 66, pp. 1-11.

[16] Gupta R, Ramnani P (2006) Microbial keratinases and their prospective applications: an overview. Applied MicrobiologyandBiotechnology.70, 21-33.

[17] Noval, J. J. and Nickerson, W. J: Decomposition of native keratinby *Streptomyces fradiae*. *J. Bacteriol.,* 77, 251 263 (1959).

[18] Cai CG, Lou BG, Zheng XD (2008) Keratinase production and keratin degradation by a mutant strain of *Bacillus subtilis*. J Zhejiang Univ Sci B 9:60-67.

[19] Syed, D. G., Lee, J. C., Li, W.-J., Kim, C.-J., and Agasar, D. (2009). Production, characterization and application of keratinase from *Streptomyces gulbargensis*. *Bioresour. Technol.* 100, 1868–1871. doi:10.1016/j.biortech.2008.09.047

[20] Bockel, S., Diamy, A. M. and Ricard, A. 1995. Optical diagnostics of active species in N-2 microwave flowing postdischarges. Surface Coatings Technol. 74-5,474-478.

[21] Vijay Kumar E, Srijana M, Kiran Kumar K, Harikrishna N & Gopal Reddy, 1996. A novel serine alkaline protease from Bacillus altitudinis GVC11 and its application as a dehairing agent, Bioprocess Biosyst Eng, 34 (2011) 403-409.

[22] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, *J Biol Chem* (1951); *193:265-75.*

[23] R.C. Dubey, D.K. Maheshwari (2002) . Practical Microbiology.

[24] Tamil Kani.P, Subha.K, Madhanraj.P, Senthilkumar.G and Panneerselvam.A,

Degrdation of chicken feathers by *Leuconostoc sp*. And *Pseudomonas microphilus*, (2012).

[25] Chang, P-Y., Hao, E., and Patt, Y. In Proceedings of the 28th annual International Symposium on Microarchitecture, (1995) pages 252-257, Michigan.

[26] Williams, C.M., C.G. Lee, J.D. Garlich and J.C.H. Shigh; Poultry sci (1991) 70:85-94.

[27] Ana Maria Mazotto, Rosalie reed Rodrigues Coelho, Sabrina Martins Lage Cedrola, Macros fabrio de Lima, Sonia Couri . Keratinase Production by Three Bacillus sop. Using feather Meal and whole feather as substrate in a submerged Fermentation (2011).