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Isolation, identification and characterization of feather degrading bacteria

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ABSTRACT

The present study deals with isolation, identification and characterization of feather degrading bacterium. The keratinolytic bacteria were isolated from feather dumped soil. The colonies showed higher keratinase production was identified as Bacillius sp, as per Bergey's manual method. Keratinase producing Bacillus sp, showed higher enzymes production. The crude filtrate showed specific activity of (>2.825)IU/mg and the protein of 4 mg/ml. Bacillus sp, produced keratinase at the pH 7 – 7.5 at the temperature of 40°C 2% feather meal as a substrate and incubation time of 96 hrs respectively which indicated that the crude keratinase enzyme produce by Bacillus sp was classified as an alkaline protease. The protein profile of Bacillus sp was analyzed in SDS – PAGE. It showed that the presence of single band which corresponds to keratinase activity. FTIR Fourier Transform Infra red Spectroscopy showed that the change in the functional group was catalyzed by unique enzymes of Bacillus sp. The keratin degrading gene sequence of isolated Bacillus sp, was analysed further studies.

Keywords: Keratinolytic bacteria, ***Bacillus sp***, FTIR, SDS-PAGE.

INTRODUCTION

Keratin is an insoluble protein macromolecule with very high stability and low degradation rate. Keratin is mainly present in hair, feather, nails, wool and horns [1]. High protein content of keratin waste can be used as a good source of protein and amino acids by systemic recycling. Recycling of feather can provides a cheap and alternative protein feed stuff. Further this can be used for animal feed and for many other purposes. However, poor digestibility of keratin is a problem in recycling [2,3]. In particular, feather from commercial poultry processing are produced in excess of million tons per year around the world [4,26] . Feathers are currently converted to feather meal using steam and chemical treatments, but these methods destroy amino acid, and require significant energy input. Alternatively the use of keratinolytic microorganisms or keratinizes has been investigated [5]. Keratinase is an extracellular enzyme used for the bio degradation of keratin. Keratinase is produced only in the presence of keratin substrate. Keratinase attacks the disulfide bond of keratin to degrade it. Some microbes have been reported to produce keratinase in the presence of keratin substrate. Keratinase producing microorganisms have the ability to degrade chicken feather, hair, nails, wool etc. [6,7]. This enzyme has been produced by bacteria ***Bacillus licheniformis*** [11, 8, 9, 10]. ***Burkholderia sp***, ***Chryseobacteriaum sp, Pseudomonas sp, Microbacterium sp*** Bernall, *et al*., 2006, was isolated and was studied with respect to different parameters. This is a potential enzyme for removing hair and feather in the poultry industry Takami *et al.*, 1992, for nutritional upgrading of feather meal and conversion of feathers into a feed protein in feed industry[3].

Keratinolytic enzymes have found to important utilities in biotechnological processes involving keratin containing wastes from poultry and leather industries, through the development of non-polluting processes. After hydrolysis, the feather was converted to feed stuffs, fertilizers, glues, films and as the source of rare amino acids, such as

serine, cysteine and proline reported by [8,7]. Feather degrading bacteria are physiologically diverse and approximately of bacterial species are unculturable because no culture methods have been established [13]. A number of keratinolytic microorganisms have been reported, including some species of fungi such ***Microsporum sp*** [15], ***Trichophyton sp*** [14] and from the bacteria ***Bacillus sp*** and ***Streptomyces*** [7,16]**.**

In this study we report the isolation of three mesophilic bacteria that produce keratinolytic enzymes, which can efficiently degrade chicken feather within 120 hours of incubation. Earlier results from the presents study involve screening of microorganism from the same soil sample from the dumping site of poultry processing plant, have reported shown the isolation of ***pseudomonas thermaerum*** Gw1, Genbank accession Gu95151. This bacterium showed proteolytic activity but not keratinolytic activity [17]. The Bacterium ***Bacillus sp*** possess high proteolytic and keratinolytic enzyme. This particular property can be used for degradation of highly stable fibrous protein, e.g. keratin, primary constituents of poultry feathers.

MATERIALS AND METHODS

Collection of soil sample
Feather dumping soils were collected from Madurai Dt. Collected soil sample was brought the laboratory and use for the isolation of microorganisms. Soil sample was weighted and kept in the closed chamber in the moist atmosphere at room temperature within 16 hours; 3-4 consecutive samples were collected.

Sterilization
All the glass wares and medium used in the study was autoclave at 15lbs pressure at 121^{o}c for 15 minutes.

Isolation of feather degrading bacteria
1gram of feather dumped soil sample transferred in 9ml of sterile distilled water. The samples were serially diluted 10^{-9} and six fold dilutions was plated on nutrient agar and incubated for 24 *hours* at 37^{o}c. The colonies were different morphology were picked and purified using streak plate method.

Characterization of keratin degrading bacterium
The isolated cultures were characterized and identified by the procedure given by (Aneja, 1994).

Preservation of isolate
Glycerol stock were prepared and stored at -80^{o}c for long term preservation. The identified pure culture slants were stored with 40% glycerol at -80^{o}c until used.

Enzyme production
The enzyme production was done by inoculating 1ml of each isolates in to 500ml Erlenmeyer flask containing 100ml liquid medium containing the following (Gram/100ml)

NH_4Cl	–	0.05
NaCl	–	0.05
K_2HPO_4	–	0.03
KH_2PO_4	–	0.04
$MgCl_2$	–	0.024
Yeast Extract	–	0.1
Raw Feather	–	1
P^H	–	7.5

The flask were shaken at 150 rpm and incubated at 37^{o}c for 5 days. After 5 days incubation the broth was centrifuged at 1000 rpm for 10minutes at 45^{o}c. The supernatant was collected for examine keratinase activity and protein content keratinase activity and protein content.

Protein determination:
Protein content was analyzed using the lowery methods with bovine serum albumin as standard protein [18].

Determination of protein molecular weight
Molecular weight of the desired protein (Keratin) is determined by SDS – PAGE.

Factors affecting keratinase production

Factors affecting the keratinase production were investigated. Factors found to be feather meal concentration, initial pH, shaking speed and culture temperature. The initial pH of the medium (pH 6.5 – 9), cultured temperature (40 - 60°C), feather meal concentration (1 -3 %), incubation time (24h , 36h, 48h, 72h, & 96h). The best keratinase producing ***Bacillus megaterium*** strain was cultured under conditions described above for 5 days to examine the bacterial growth and its keratinase production.

FTIR

Fourier transform Infra red spectroscopy (FTIR) is an powerful tool for identifying types of chemical bonds in a molecules by producing and infra red absorption spectrum that is like a molecular " finger print".

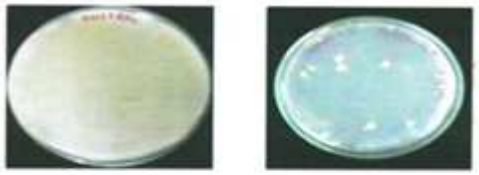


Plate1. Isolation of Keratinolytic Micro Organisms

Plate2. Pure Culture of Isolates (A1-A8)

Fig – 1 & 2 : keratinolytic isolates on Nutrient agar Medium

Table – I: Morphological characterization

1.	Colony properties	On nutrient agar colonies, are circular, smooth, round, waxy, slight white, mucoid produce no pigment
2.	Gram's staining	Gram positive, rod
3.	Motility	Motile.

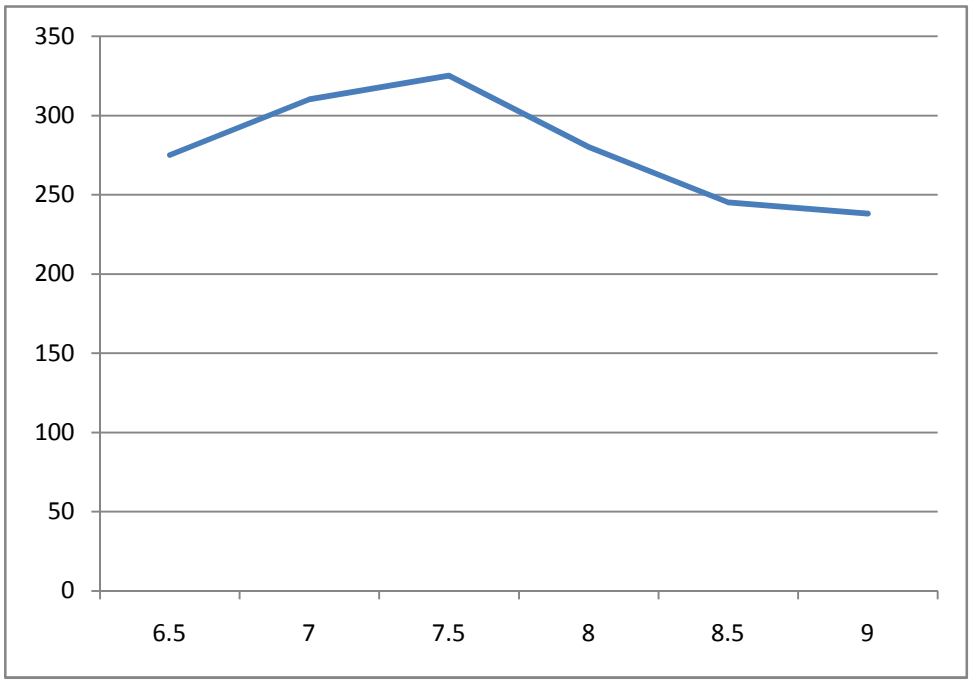
Table II: Bio- chemical characterization

S.NO	BIO-CHEMICAL TEST	*Bacillus sp*
1	Indole production Test	Negative
2	Methyl Red Test	Positive
3	Voges proskaeur Test	Positive
4	Citrate Utilization	Positive
5	Triple Sugar Iron Agar Test	Positive/Presence of Air Bubbles
6	Catalase	Positive

Table – III: Effect of various P^H on keratinase production

S. No	pH	Keratinolytic activity (u/ml)
1	6.5	275
2	7	310
3	7.5	325
4	8	280
5	8.5	245
6	9	283

Fig - III. Effect of various pH on keratinase production



Y – axis: Keratinolytic activity [u/ml]

Table – IV: Effect of temperature on keratinase production

S. NO	Temperature	Keratinolytic activity (u/ml)
1	40°C	299
2	45°C	260
3	50°C	252
4	55°C	242
5	60°C	210
6	65°C	197

RESULTS

In the present investigation, the keratin utilizing bacteria were isolated from the feather dumped soil. The microorganisms responsible for the degradation of native keratin were identified by the morphological, biochemical and cultural characteristics.

Isolation of the bacterial species
The isolates formed light yellow color colonies on nutrient agar medium (Fig – 1 & 2)

Morphological characteristics and biochemical characterization:
The morphological and biochemical characters were listed in Table – I and Table – II.

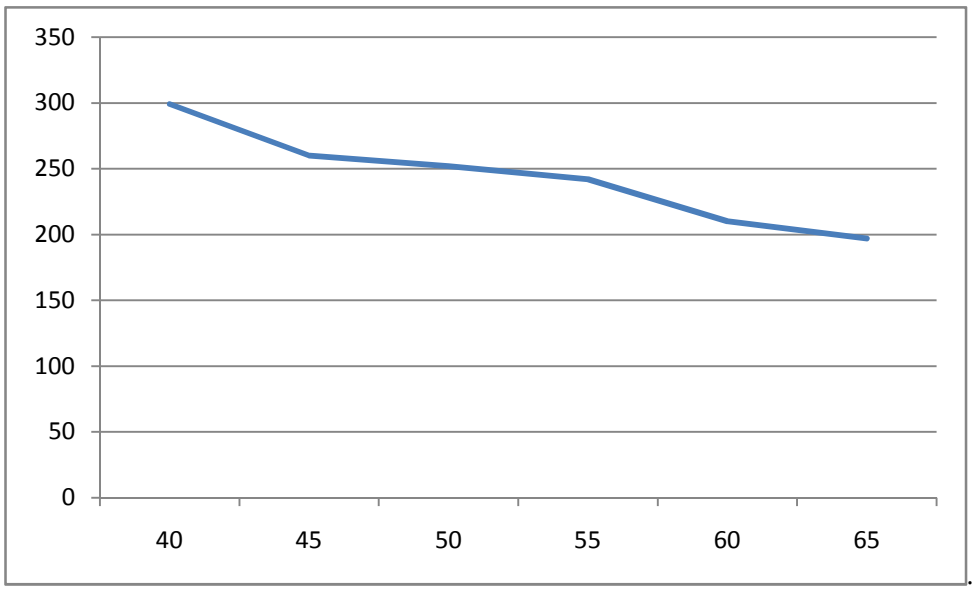
Optimization of media pH for keratinolytic activity
Keratinolytic activity were checked at different media pH (6.5-9.0) results were tabulated (Table – III & Fig - III). The keratinolytic activity was observed at the pH (7.5). Maximum enzyme production was observed between pH 7 and 8. High enzyme production was achieved at the pH 7 and 7.5.

Effect of temperature on keratinolytic production
Effect of temperature on keratinolytic activity was investigated under acid condition. Maximum keratinolytic activity was achieved at 40°C (Table – IV & Fig - IV). Keratinolytic activities of various substrate concentrations (1-3g) were investigated. Maximum keratinolytic activity was examined in 2g of substrate concentration were used for ***Bacillus sp*** (Fig – V & Fig - VI).

Effect of incubation time of keratinase production

Effect of time on keratinolytic activity was investigated. Maximum keratinolytic activity was achieved at 96 hours. (Table – V & Fig - VII).

Fig - IV. Effect of various Temperatures on keratinase production



Temperature (°C)

Y – axis: Keratinolytic activity [u/ml]

Fig – V: Different feather substrate treated with Biomass

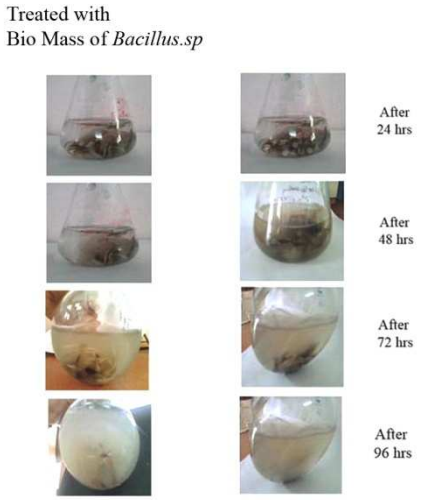
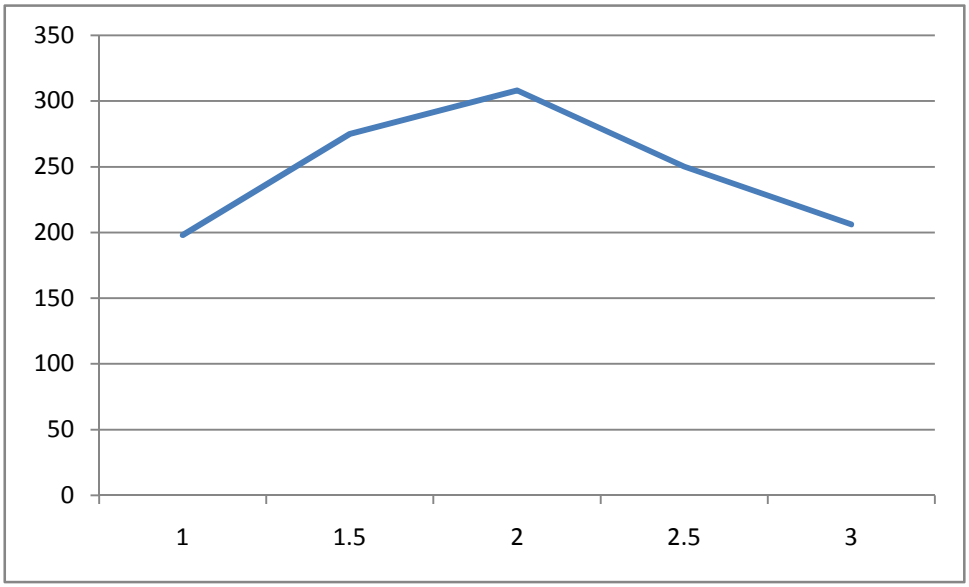


Plate: Degradation of Keratin waste by Bacillus.sp

Fig - VI Effect of various substrate concentrations on keratinase production



Substrate Concentrations (g)
Y – axis: Keratinolytic activity [u/ml]

Table – V: Effect of incubation time of keratinase production

1	24 hours	140
2	48 hours	165
3	72 hours	190
4	96 hours	302
5	120 hours	250

Fig – VII: Effect of Incubation time on keratinolytic activity

X – axis: Incubation time (Hours)
Y – axis : Keratinolytic activity (u/ml)

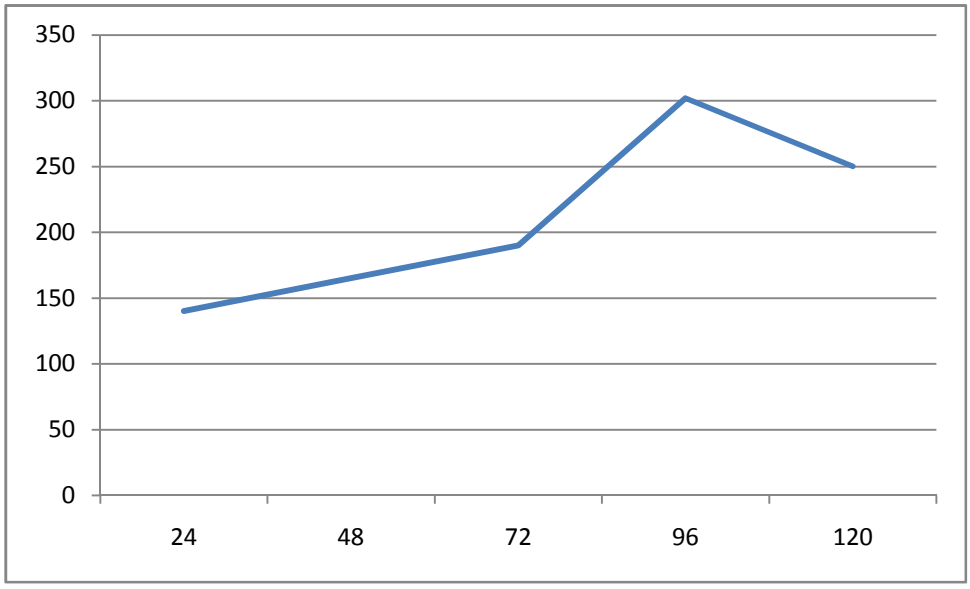


Fig – VIII: Electrophoretic separation of Keratinase Enzyme of *Bacillus sp*

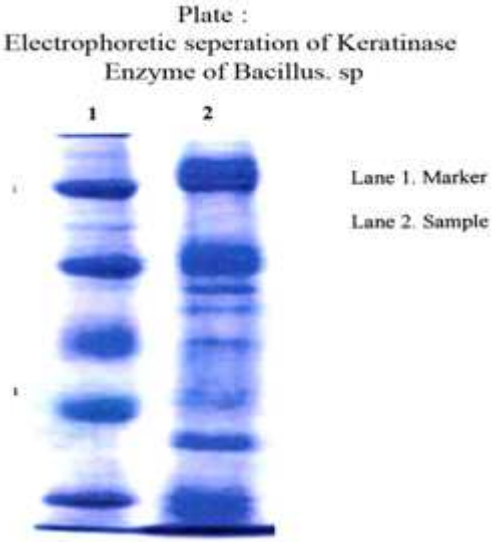
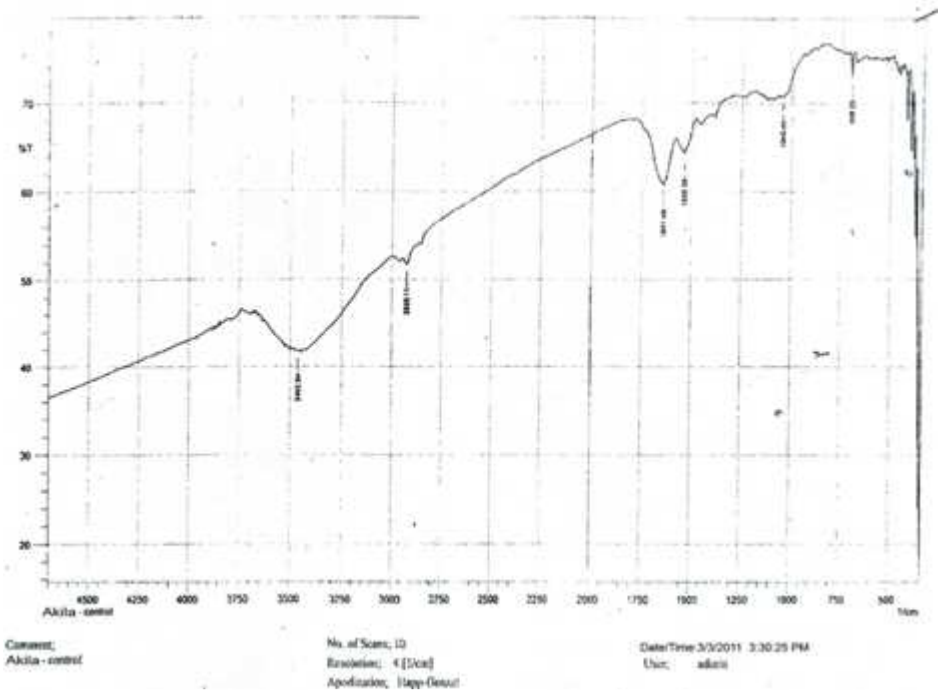


Fig – IX: FTIR pattern of *Bacillus sp*



DISCUSSION

In the study the keratinase enzyme producing ***Bacillus sp*** was isolated optimum conditions for maximum enzyme production in feather minimum broth medium containing keratin feather, and degradation of feather by selected isolate was studied. These colonies were tested for their ability to grow on feather minimal isolate-1 showed higher keratinolytic activity compared with other isolate. The morphological and biochemical characters were compared with Bergey's manual of determinative bacteriology. The isolate A1 was examined gram positive rod , spore forming and non motile, negative results for indole production test, methyl red test, voges proskauer test and positive result for citrate test, TSI, catalasee test as per the Bergey's manual the isolate strain were identified belonging the ***Bacillus sp***. Similar result was reported by [10], found a straight rod shape aerobic and endosspore forming bacterium appeared in single or in chain catalase positive and gram variable classified as ***Bacillus sp***. This bacterium isolated from high temperature poultry waste digest and able to degrade feather keratin when using feather as a primary source of carbon and energy. Atalo and Gashae 1993 described thermophillic ***Bacillus sp***, produce protease to degrade various s fibre proteins feather hair sheep skin horn.

In addition [2] reported a rod shaped and spore forming bacterium identified as ***Bacillus sp***, produce a thermostable alkaline protease to degrade human hair. described that a keratin degrading strains of thermophilic anaerobic bacteria, rod shaped with an outer sheath like structure of 2.02 - 20µm long occurred singly or in pairs. Gram-Negative and no endospore forming [20] reported *Bacillus sp*. Where able to degrade keratin substrates from human hair, cow corn and cow hooves in vitro presently bacterial growth and keratinase producing by *Bacillus* during cultivation was observed. The organisms showed effective degradation till 136 hours where its stationary phase ends. Similar results were found in other *Bacillus* Strains [19, 21]. Depending upon the keratinolytic activity, keratinase enzyme producing *Bacillus sp* was cultured in feather meal broth. The crude filtrate showed a specific activity of 72.875 IU/mg and the protein content of 4mg/ml were as *Bacillus sp* 511 showed specific activity of 242 U/mg after 48 hrs of incubation *Bacillus* sp showed specific activity of 198 U/mg after 48 hrs of incubation. *Bacillus* sp 717 showed specific activity of 198 U/mg after 48 hrs of incubation. Based up on the activity *Bacillus sp* 511 was selected for the further studied. This study was reported by [22].

Several external factors affect the production of enzymes. These factors including varation in pH, temperature, incubation time and substrate concentration. Changes in pH affect the enzyme production but it may also change the properties of substrate. Keratinase enzymes production was observed at the pH range from 6.5 – 9.0 from for ***Bacillus sp*** *(*1) respectively. Maximum enzyme production was achieved at medium pH 7.5 for ***Bacillus sp*** (AI). Below and above the production rate was decreased gradually. Similar result was also observed by [23]. Maximum keratinase production was obtained when an initial pH of medium was 7.5 higher growths were observed. When initial pH at 6.5, 7.0 [19] found to optimum pH for keratinase production by *Bacillus sp* 00IA at pH 7.5 [7] also reported the highest keratinase production by *Bacillus sp* Pwd – 1 was at an initial pH of 6.5.

Temperature has great impact on enzyme production. A higher temperature generally results in an increasing enzyme production. If the temperatures rises beyond a certain point, however the enzyme production eventually leveled out than decline rapidly because the destroying of organism by high temperature. Presently, optimum temperature for keratinase production observed at 40°C, no keratinase production was observer at 50°C and 65°C because of on absence of bacterial cell growth at such high temperature, were as the highest bacterial growth and keratinase production were found at the incubation temperature of 37°C (Dakron *et al.*, 2001). Poor growth and 59% less keratinase production were found at 25°C compare with *Bacillus sp* pwD – 1 the maximum growth was reported at 50°C. While the maximum enzyme production was obtained at 40°C [7].

The amount keratinase production depended on substrate concentration and cultivation conditions. In the present study highest keratinase production was observed on substitution with 2% feather meal. Similar finding was found with high feather concentration (2% b) for protease production. were as highest keratinase production was obtained at 2% feather meal for ***Bacillus sp***, Cheng *et al.*, 1995 also reported that 2% feather have the highest keratinase activity for ***Bacillus*** Pwd- 1. The time required to attain the maximum enzyme production by ***Bacillus Sp*** was determined keratinase production reached maximum at 96 hrs of incubation. were as maximum keratinolytic was obtained by cultivation of ***Bacillus sp*** in feather meal medium after 24 hrs [22].

The protein profile was analysed in SDS – PAGE. In the present study the keratinase enzyme isolated from Bacillus was analysed by SDS – PAGE 10% polyacrylamide slab. If showed that the presence of multiple bands because in all the n causes the total protein content of the samples were moderately high. Similar work was done by [23]. Zymography analysis in SDS – PAGE showed a single band which corresponded keratinase activity was observed by zone in the zymogram. Similarly a single clear band corresponding to proteolytic activity was observed [23]. A unique structure of keratin makes it very resistant to proteolytic digestion . The resistant only to super coiled helical structure of poly peptide chain but also to the strength of enter molecular disulfide bonds and other molecular interactions. Feather hydrolyzed by mechanical and chemical treatment. Alternatively keratinase is an enzyme which was secreted by many microorganism that hydrolyzing keratin in to smaller molecular [23].

Degradation of the organic type of contaminant by microbes leads to complete by releasing carbondioxide and process known as mineralization. Thus use of microorganisms is an obvious choice in the biodegradation of environmental pollutant [23]. Keratin degrading or enzymatic biodegradation better alternative to improve nutritional value of poultry feather and reduce environmental waste. In conclusion a bacterial strain designated as *bacillus sp* was isolated from Pasumalai area Madurai. This bacterium was grown in basal media with feathers meal as its primary source carbon, nitrogen, sulphur and energy. It was able to produced keratinase and could be applied for feather degradation in to feather hydrolysate. Its keratinase production was maximal in a substrate pH 7.5 and under cultivation condition at the temperature of 35°C and the incubation time of feather were obtained by *Bacillus sp* indicated that the absence on

contaminants. The protein profile in SDS-PAGE showed the presence of multiple bands because the high total protein content of the sample. Zymography analysis showed a single band which corresponds to keratinase activity Fourier transform infrared spectroscopy (FTIR) showed that the functional group of the parent molecules was changed into intermediates by oxidation and reduction reaction catalysed by unique enzymes of *Bacillius sp*. Therefore the newly isolated *Bacillus sp* shows potential use in biotechnological process that involve degradation and utilization of feather keratin (Fig – IX). Also this isolate can be expected to improve the nutritional value of animal to feeds that contain feather and other keratin or waste from poultry processing.

However, further elucidated that for the evolution of biotechnological application of the keratinolytic protease from selected bacteria requires more detailed understanding of the factors that enable this enzyme for complete degradation of native keratinous substrates. Therefore additional researches have to be done for purification and characterization of purification and characterization of keratins, studying the kinetics of enzyme, testing for the range of substrates, effect of inhibitors and enhancing the activity of keratinase submerged state fermentation and large scale production of keratinase.

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REFERENCES

[1]. Onifade, A.A., A1-Sane *et al*., *Biosource Technology* (**1998**), 66, 1-11.
[2]. Takami,H., N.Saatoshi, R.Aono and K.Horikoshi., *Biosci –Biotechnol. Biochem*. (**1992**):56:1667-1669.
[3]. Williams,C.M., C.G.Lee, J.D.Garlich and J.C.H.Shigh; *poultry sci* (**1991**) 70 :85-94.
[4]. Shighn J.C.H, *poultry science* (**1993**) .72:1617-1620(2).
[5]. Wang, X., Parsons,C.M., *poultry science* (**1997**).76,491-496.
[6]. Gradisar. A *Animal feed science and Technology* (**2005**).126,135-144.
[7]. Cai, C.and X.Zheng, *J.Ind.Microbial Biotechnol*., (**2009**) 36:875-883.
[8]. Ramnani,P, R,singh *can J.Mechanical*.,(**2005**):51:191-196.
[9]. Korkmaz P.Letourneau, Shign, *Applied and environmental microbiology* (**2004**) 65, 2570-2576.
[10]. Williams, C.G.Lee and J.D.Garlich, *poultry science*.(**1990**) 76,491-496.
[11]. Zerdani,I., M.Faid and A.Malki, *Afr .J.Biotechnol*.,(**2004**):3:67-70.
[12]. Bernal, C.Cairo, H.Takami, AL.Sane *Enzyme and microbial technology* (**2006**).38, 49-54.
[13]. Amann, A.A.Onifade, H.Satoshi, C.M.Williams, *Microbiology.Rev* (**1995**), 59:143-169.
[14]. Anbu, P.,A.Hilda,Wang, Parson Int, *Biodeterior biodegrade* (**2008**).,62:287-292.
[15]. Essien, *A Microbiological Et immunoloical .Hungarica* (**2009**),56;61-69.
[16]. Macedo.S G.Lee,J.D.Garlich,and J.C.H.Shigh. *poultry sci* (**2005**):70:85-94.
[17]. Gaur,S. and S.Agrahai *The open Microbiology,J* (**2010**);vol.3
[18]. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, *J Biol Chem* **(1951)***; 193:265-75.*
[19]. Atalo, K. and Gashe, B.A., *Biotechnol Lett* (**1993**)15, 1151–1156.
[20]. Lal, R., H.M. Hassan and J. Dumanski Columbus (**1999**), Ohio: Malone Battelle Press.
[21]. Chang, P.-Y., Hao, E., and Patt, Y. In *Proceedings of the 28th Annual International Symposium on Microarchitecture*, (**1995**) pages 252-257, Michigan.
[22]. Veslava Matikeviciene, Danute Masiliuniene, Saulius Grigiskis, *Proceedings of the 7th International Scientific and Practical Conference* (**2009**). *Volume 1*
[23]. Bo Xu, Qiaofang Zhong, Xianghua Tang, Yunjuan Yang and Zunxi Huang, *African Journal of Biotechnology* (**2009**) Vol. 8 (18), pp. 4590-4596,
[24]. J.H. Bin, A.L. Lei, W. Yu, *Enzyme and microbial technology* (**2009**), 45, 282 – 290.
[25]. Aneja, K.R., (**1994**). Biochemical activities of microorganism. Experiments in Microbiology, Plant pathology, Tissue culture and Mushroom production technology. New Age International publishers. IIIrd Edn. 245 – 275.
[26]. Shrooyen, D and shine, P *Poultry Science* (**2001**); 194: 114 - 123