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# Industrial application of keratinase and soluble proteins from feather keratins

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***Abstract:*** *Feather keratin is highly resistant to degradation, but some keratinase producing microorganisms can easily degrade these insoluble keratins. These keratinase producing species have an important apnplication in removal of poultry waste and recycled into valuable byproduct. Bacillus sp was screened from soil samples of slaughter house and poultry farm area using azokeratin medium. Highest keratinase activity (122.5 KU ml$^{-1}$) was observed at 8.0 pH. Submerged fermentation was carried out at 8.0 pH up to 5$^{th}$ days. On 4$^{th}$ day enzyme production was highest (140 KU ml$^{-1}$) with 1% feather (w/v).Crude protein content was high on day 5, around 1.44 mg ml$^{-1}$. 75% of filtrate was found to be crude protein. The molecular weight of this keratinase was 32 kDa by SDS-PAGE. The crude protein from feather has of high nutrient value and could be used as animal feed for livestock and fish feed in aquaculture.*

***Key word:*** *Feather keratins, Degradation, Keratinase, Crude protein, Animal feed*


## Introduction

Animal wastes have been used as nutrient sources for crop production. During the last three decades, researches have been conducted to improve the agronomic utilization of animal wastes, including poultry waste, slaughter waste and human waste. Feather waste, resulting in large quantities as byproduct of poultry farms processing, are pure keratin proteins. Poultry feather keratin is highly resistant to degradation. Over 5% of the chicken has feathers with alpha or beta keratin. This material can be recycled into useful products and it would have great commercial value. A microbial inoculum might convert waste feathers into supplements for digestible feed for poultry, livestock and fish and can also be utilized for the production of biogas. Any liquid nutrient residues from feather composting that are high in nitrogen could be used for aquaculture and hydroponics crops (Ichida *et al*., 2001).

Keratins are insoluble proteins from feathers, wool, hooves, scales, hair, nails (hard keratins) and stratum corneum (soft keratins). These proteins which belong to the scleroprotein groups are compounds that are extremely resistant to the action of physical, chemical and biological agents. Mechanical stability and high resistance to proteolytic degradation of keration are due to their disulfide bonds, hydrogen bonds, salt linkages and cross linkings (Kaluzewska *et al*., 1991).

The feathers which are hydrolysed by mechanical or chemical treatment can be converted to feed-stuffs, fertilizers, glues and foils. These are also used for the production of aminoacids and peptides. Current commercial production of feather meal involutes, treatment at elevated temperature and high pressure. This process in addition to being energy intensive results in the loss of some essential aminoacids (Papadopoules *et al*., 1986). Because of environmental considerations the use of keratinolytic enzymes in the production of amino acids and peptides is becoming attractive for biotechnological applications. Due to insoluble nature of keratin, it is resistance to enzymatic digestion by plants, animals and many known microbial proteases. Therefore the keratinase producing microorganisms have been described having the ability to degrade feather. They are general species of fungi, actinomyces and bacteria *viz*., *Doratomyces microsporus*, *Aspergillus* sp, *Alternaria radicina*, *Trichurus spiralis, Stachybotrys atra*, *Onygena* sp, *Absidia* sp, *Rhizomucor* sp (Friedrich *et al*., 1999), *Streptomyces pactum*, *S. albs*, *S. thermoviolaceus*, *S. fradiae* (Noval and Nickerson, 1959), *S.thermonitrificans* (Mohamedin.,1999), *Flavobacterium pennavorans, Bacillus* sp (Suntornsuk and Suntornsuk, 2003), *Stenotrophomonas* sp (Yamamura *et al*., 2002), *Bacillus licheniformis* and *B. pumilus* (Nitisinprasert *et al*., 1999) and *Vibrio* sp (Sangali and Brandelli, 2000).

Keratinase producing microorganisms have the important industrial application in fermentation technology. Submerged fermentation of poultry waste by microorganism producing keratinase helps in the conversion of non-soluble keratin (feather) into soluble protein or polypeptide (Suntornsuk and Suntornsuk, 2003).These protein byproduct may be used as animal and livestock feed, and as leather filling agents (Sastry *et al*., 1986). Keratinase has also emerging application in dehairing process in leather industry instead of sodium sulphides (Alexandre *et al*., 2005) and also used as a detergent to remove strains on cloth (Gessesse *et al*., 2002). Valorization of keratin containing wastes like feathers from poultry farms and hair from leather industries may have the potential in development of non polluting processes. The scope of this work is to degrade the poultry feather wastes (insoluble protein) to soluble protein.

## Materials and Methods

Soil samples were collected from slaughter house and poultry farm area. Keratinase producing bacteria was isolated from zone formation in casein agar medium and identified as *Bacillus* sp Keratinase was confirmed using azokeratin (soluble keratin) medium (Suntornsuk and Suntornsuk, 2003).

**Keratinase activity and feather degradation at different pH:** Chicken feather 250 mg was transferred to 25 ml of basal medium ($NH_3Cl$-0.5 $gl^{-1}$, $K_2HPO_4$-0.3 $gl^{-1}$, $KH_2PO_4$-0.4 $gl^{-1}$, MgCl-0.24 $gl^{-1}$, peptone-0.2 $gl^{-1}$) and adjusted the pH 6.0 to 10.0 with 1N NaOH in each flask. Inoculate at 5% inoculum with the initial cell count of 5 x $10^7$ cells $ml^{-1}$ and incubated for 5 days at 37°C in rotor shaker with 120 rpm. At the end of the 5th day of incubation, the culture was filtered and the dry weight of the feather was observed. The percentage of feather hydrolyzed at each pH was calculated.

**Estimation of enzyme activity :**

**Assay for keratinase:** Keratinase activity was followed by the modified method (Yamamura *et al.*, 2002). The keratinolytic activity of the mixture containing 2 ml azokeratin (1% w/v) and 0.5 ml suitably diluted enzyme was carried out at 45°C for 30 min. The enzymatic reaction was stopped by adding with 2.5 ml of 10% TCA (Trichloro acetic acid) and then allowed to settle for 30 min and then filtered. To 1 ml of the filterate, 5 ml of 0.5 mM sodium bicarbonate solution and 0.5 ml of diluted Folin-Ciocalteau reagent were added. After the reaction mixture was incubated for 30 min, the absorbance was measured at 660 nm using spectrophotometer. Simultaneously a blank was read using the same steps except that 10% TCA was added prior to the addition of enzyme. Results were expressed as Keratinase units (KU $ml^{-1}$) of enzyme.

**Protein estimation:** The amount of protein in the culture filtrate was quantified by the method explained by Lowry *et al*. (1951). Mixed 1 ml of sample with 5 ml of freshly prepared alkaline copper sulphate and incubated at room temperature for 10 min.To this 0.5 ml of Folin-ciocalteau's reagent was added and incubated at room temperature for 20 min and the absorbance was measured at 660 nm wavelength. The blank was prepared using the same procedure without sample. The protein content was estimated by calibration with the standard graph.

**Total Kjeldhal nitrogen (TKN) estimation:** Total nitrogen content of the sample was estimated by the method explained by Jayaraman (2000) and on this basis total amount of protein present in the sample was worked out. Nitrogen (as protein) in the feather degraded broth was converted to ammonium sulphate by digesting with concentrated $H_2SO_4$ in the presence of catalyst mixture. The ammonia liberated by the addition of excess caustic soda was distilled into an absorbent solution and was estimated by the titration by HCl solution (1/70 N). The ammonia ($NH_3$) liberated was proportional to the protein aminoacid content of the sample used. This titrated value was multiplied by the TKN standard value (Total Kjeldhal Nitrogen) 6.25.

**Characterization and identification of molecular weight of the keratinase:** Culture filtrate was centrifuged at 10,000 rpm for 15 min and the supernatant was precipitated by ammonium chloride or acetone. Then the precipitate was dialyzed. Around 50 µl of the precipitate was run on SDS-PAGE with standard marker.

## Results and Discussion

The practical use of keratinase producing microorganisms is being explored in applied microbiology where there is great need for active degraders of feather keratin. *Bacilus* sp can produce keratinase which can degrade the feather keratin to soluble crude proteins. Keratinase can actively degrade the feather at pH 8.0 when incubated for 5 days. At this pH, activity of keratinase was maximum 122.50 (KU $ml^{-1}$) which reduced further with higher pH (Fig. 1). These results were in line with (DeToni *et al*., 2002) indicating that keratinase produced by *Bacilus* sp could be classified as an alkaline protease and was most active under neutral as basic condition. *Kocuria rosea* Keratinase has the optimum at alkaline pH and 40°C resistant to denaturing or reducing agents such as dithiotreitol and 2-mercaptoethanol (Bernal *et al.,* 2006).

For the production of keratinase and degradation of feather, 1% with basal medium and feather 1% with 5% inoculam was used and maintained in a shaker at the rotation speed of 120 rpm/min at 37°C.Suntornsuk and Suntornsuk (2003) used yeast extract in low concentration and observed 100 µg $ml^{-1}$ of keratinase on 5th day of incubation with only 85% of feather degradation. The growth, feather degradation and keratinase production of *Bacillus* sp FK46 were optimum at an incubation temperature of 37°C. *Bacilus* sp keratinase can degrade the feather keratin to soluble crude protein. Keratinase activity was estimated at different interval (1, 2, 3, 4 and 5 days) at pH 8.0 (Fig. 2). On 5th day maximum protein content (1.44 mg $ml^{-1}$) was estimated (Fig. 3). Mohamedin (1999) also stated that high protein content reached at 72 hr of incubation and decreased thereafter. After 5th day of incubation, *Bacillus* sp, can utilize the soluble protein in that medium. Therefore the culture filtrates was collected after 5th day by centrifugation to remove bacterial cells and was purified by column chromatography. The soluble protein determined by HPLC analysis by Wang *et al.* (2003), also showed that feather keratin was degraded at specific cleavage sites to produce a stable 18 KDa protein.

Exact aminoacid content in the crude protein by aminoacid estimation method would not be estimated because most of the crude proteins were poly peptides. Murphy and King (1986) reported that aminoacid composition of feather was in lysine, phenyl alamine, tyrosine, alanine lysine and serine. Micro Kjeldhal nitrogen estimation used to estimate the total protein content of the crude protein from the hydrolyses revealed that 75% of the feather keratin was converted into crude soluble protein at 5th day (Fig. 4).These finding indicated that the crude protein may be used as animal and livestock feed. The molecular weight of the keratinase was characterized and identified by SDS-PAGE as 32 kDa with standard marker (Fig. 5).

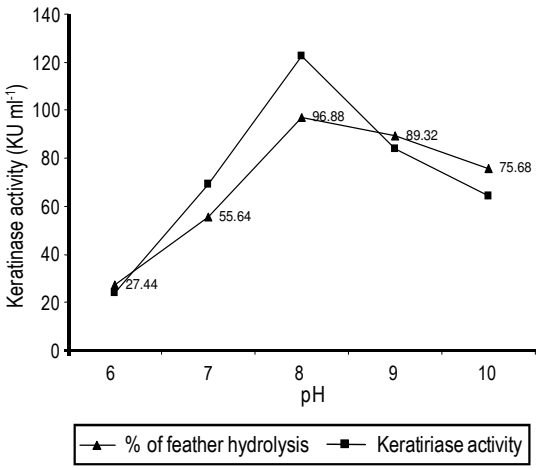


**Fig. 1:** Keratinase activity and percentage of feather hydrolysis at different pH levels

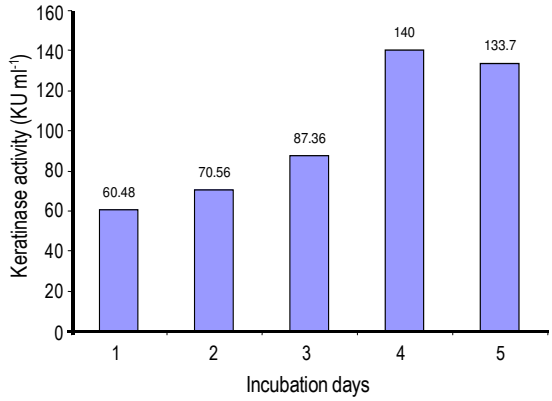


**Fig. 2:** Keratinase activity on different day of incubation

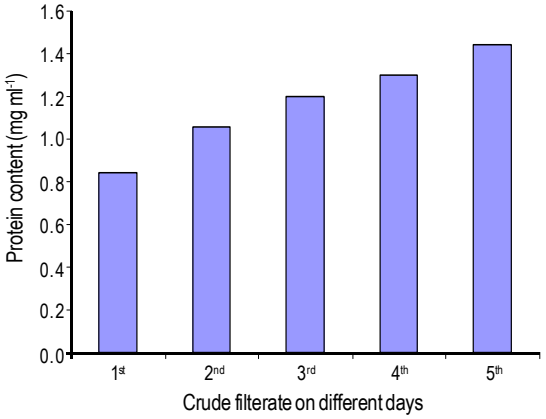


**Fig. 3:** Protein content of the crude filtrate for 1% (w/v) feather

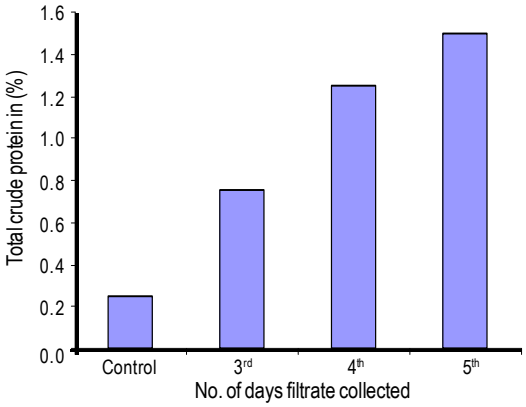


**Fig. 4:** Percentage of crude protein from TKN value

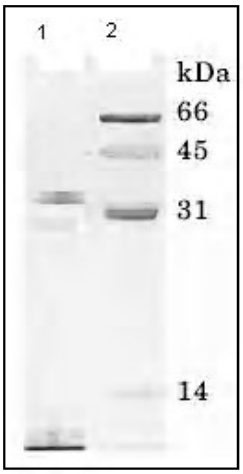


**Fig.5:** SDS-PAGE: Lane 1 = Keratinase enzyme 32 kDa, Lane 2 = Bovine serum albumin, ovalbumin, serine protease and chymotrypsin

We can also use this feather as a substrate to produce high amount of keratinase. Keratinase can degrade all the protein molecules, so it may also be used detergent purpose. This enzyme can be an alternative to sodium sulfide, the major pollutant from tanneries, and may completely replace it. Its unique nonactivity upon collagen enhances its industrial potential (Alexandre *et al.,* 2005). It is therefore easy to convert the feather keratin into soluble crude protein through microbial fermentation technology by using pilot scale bioreactor (Singh, 2007) and soild state aerobic and anaerobic reactors to yeild byproducts (Rajesh Banu *et al*., 2006). Besides pollution problem posed by poultry waste can also be reduced by this technology.

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## References

Alexandre, J.M., O.B. Walter, G. Renata, D. David, P.H. JoaoAntonio and T. Carlos: Novel keratinase from *Bacillus subtilis* S14 exhibiting remarkable dehairing capabilities. *Appl. Environ. Microbiol.*, **71**, 594-596 (2005).

Bernal, C., J. Cairo and N. Coello: Purification and characterization of a novel exocellular Keratinase from *Kocuria rosea*. *Enzyme Microb. Technol.*, **38**, 49-54 (2006).

DeToni, C.H., M.F. Richter, J.R. Chagas, J.A. Henriques and C. Termignoni: Purification and characterization of an alkaline serine endopeptidase from a feather degrading X*anthomonas maltophilia* strain. *Can. J. Microbiol.*, **48**, 342-348 (2002).

Friedrich, J., H. Gradisar, D. Mandin and J.P. Chaumont: Screening fungi for synthesis of keratinolytic enzymes. *Lett. Appl. Microbiol.,* **28**, 127-130 (1999).

Gessesse, A., H.K. Rajni, B.A. Gashe and Bo Mattiasson: Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. *Enzyme Microbial. Technol.*, **6250**, 1-6 (2002).

Ichida, J.M., L. Krizova, C.A. Lefevre, H.M. Kerner, D.L. Elwell and E.H. Jv Burtt: Bacterial inoculum enhances keratin degradation and biofilm formation in poultry compost. *J. Microbiol. Methods,* **47**, 199-208 (2001).

Jayaraman, J.: MicroKjeldhal nitrogen estimation. Laboratory manual in biochemistry. New Age International Publishers, New Delhi. pp. 75-78 (2000).

Kaluzewska, M., K. Wawrzkiewicz and J. Lobarzewski: Microscopic Examination of keratin substrates subjected to the action of the enzymes of *Streptomyces fradiae*. *Int. Biodeterioration*, **27**, 11-26 (1991).

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275 (1951).

Mohamedin, A.H.: Isolation, identification and some cultural conditions of a protease – Producing thermophilic *Streptomyces* strain grown an chicken feather as a substrate. *Int. Biodeterioration Biodegradation,* **43**, 13-21 (1999).

Murphy, M.E. and J.R. King:Composition and quantity of feather sheaths produced by white-crowned sparrows during the postnuptial molt. *Auk.,* **103**, 822-825 (1986).

Nitisinprasert, S., W. Pornuirum and S. Keawsompong: Characterizations of two bacterial strains showing high keratinase activities and their synergism in feather degradation. *Kasetsart J. Nat. Sci.,* **33**, 191-199 (1999).

Noval, J. and W.J. Nickerson: Decomposition of native keratin by *Streptomyces fradiae*. *J. Bacteriol.*, **77**, 251-263 (1959).

Papadopoules, M.C., El. Boushy, A.R. Roodbeen and E.H. Ketalaars: Purification and characterization of a keratinolytic serine proteases from *Streptomyces albidoflavus*. *Appl. Environ. Microbiol.*, **65**, 2570-2576 (1986).

Rajesh Banu, J., S. Kaliappan, M. Rajkumar and Dieter Beck: Treatment of spent wash in anaerobic mesophilic suspended growth reactor (AMSGR). *J. Environ. Biol.*, **27**, 111-117 (2006).

Sangali, S. and A. Brandelli: Feather keratin hydrolysis by a *Vibrio* sp strain kv2. *J. Appl. Microbial.,* **89**, 735-743 (2000).

Sastry, T.P., P.K. Sehgal, B. Gupta and Mahendra Kumar: Solublised keratins as a Novel filler in the retaining of upper leather. *Leather Science.*, **33**, 345-359 (1986).

Singh, Pratibha: Sequential anaerobic and aerobic treatment of pulp and paper mill effluent in pilot scale bioreactor. *J. Environ. Biol.*, **28**, 77-82 (2007).

Suntornsuk, W. and L. Suntornsuk: Feather degradation by *Bacillus* sp FK 46 in submerged cultivation. *Bioresour. Technol.*, **86**, 239-243 (2003).

Wang, J.J., H.E. Swaisgood and J.C. Shih: Production and characterization of bio-immobilized keratinase in proteolysis and keratinolysis. *Enzyme Microb. Technol.*, **32**, 812-819 (2003).

Yamamura, S., Y. Mosita, Q. Hasan, K. Yokoyama and E. Tamiya: Keratin degradation : A cooperative action of two enzymes from *stenotrophomonas* sp. *Biochem. Biophl. Res. Commun.*, **294**, 1138-1143 (2002).