Int J Pharm Bio Sci 2013 Jan; 4(1): (B) 117 -126

Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

UTILIZATION OF BIODEGRADABLE KERATIN CONTAINING WASTES BY ENZYMATIC TREATMENT

R. KANCHANA

Department of Biotechnology, Parvatibai Chowgule College of Arts& Science, Margao,Goa-403 004, India

ABSTRACT

Two novel feather-degrading fungi belonging to *Chrysosporium and Microsporum* genus were isolated from poultry waste in Goa, India. The fungi produced extra cellular keratinolytic enzyme in a feather meal broth using chicken feather as a sole of carbon and nitrogen source. Maximum enzyme activity was obtained within 96 h (150 U/ml and 90 U/ml for *Chrysosporium and Microsporum* respectively) of cultivation at pH 7.0, 30^{o}C. The optimal conditions for the keratinolytic activity of both enzymes were found to be pH 9.0 and temperature 50°; however the enzymes showed stability over a broad range of pH between 7.0 -10 and temperature 30°C - 70°C. In addition, both the strains were able to completely degrade feather within a period of 96 h.

KEY WORDS: **Feather, feather degradation, keratinase, keratinolytic activity, poultry wastes**

***Corresponding author**

R. KANCHANA
Department of Biotechnology, Parvatibai Chowgule College of Arts& Science, Margao,Goa-403 004, India

INTRODUCTION

Feather wastes are generated in large quantities as a by-product of commercial poultry processing. Feathers represent 5-7% of the total weight of mature chickens. Feathers are made up primarily of keratin, which is also found in the claws and armour of reptiles and the hooves, horns, hide, hair and nails of mammals[1]. Keratin is a fibrous and insoluble structural protein extensively cross-linked with disulphide, hydrogen and hydrophobic bonds, resulting in mechanical stability and resistance to common proteolytic enzyme such as pepsin, trypsin and papain. These feathers constitute a sizable waste disposal problem. Several different approaches have been used for disposing of feather waste, including land filling, burning, natural gas production and treatment for animal feed. Most feather waste is land filled or burnt which involves expense and can cause contamination of air, soil and water[2]. Feathers are currently used to produce feather meal through thermal processing, resulting in a low nutritional value product. Feather hydrolysates produced by microbial keratinases have been used as additives for animal feed. In addition, keratin hydrolysates have potential use as organic fertilizers, production of edible films and rare amino acids[3]. Keratinases can be produced by many microorganisms such as bacteria, *Actinomyces* and fungi. Till date most of purified keratinizes known cannot completely solubilise native keratin, their exact nature and uniqueness for keratinolysis is still not clear[4]. There is always a requirement of isolation of enzymes from new sources to meet the industrial and environmental demand. Utilization of these potential keratin degraders will definitely find biotechnological use in various industrial processes involving keratin hydrolysis. It would also solve the waste disposal problem of poultry waste and recycling of keratinacious waste would be beneficial financially and environmentally[5]. Among the microbes that cycle this protein in nature, keratinophilic fungi are very common and the most diverse. If keratinophilic fungi were not there to cycle this highly stable protein (keratin), then one can imagine the quantity of keratin that would have accumulated on earth, since a vast quantity of keratin is shed by the vertebrates. Indian soils contain many more keratinophilic fungi than those presently recorded, and there is need for further taxonomic and ecological studies of this interesting group of organisms[6]. The present work is thus carried out to identify and explore sources of keratinases producing fungi from soil of feather dumping sites from Goa, India.

MATERIALS AND METHODS

Isolation of Keratinophilic Fungi

The soil samples were collected from the feather processing areas. Hair baiting technique was followed for the isolation of keratinophilic fungi[6]. The sporulated cultures were transferred to Potato Dextrose agar plates.

Feather meal powder preparation

Feather meal powder was prepared according to the method described by Agrahari & Wadhwa (2010)[7]. In brief**,** chicken feathers were washed extensively, defatted and dried in hot air oven. The dried feathers were pulverized and the powder was used as feather meal.

Screening on casein agar plates

The selected strains from baiting method were spread plated on casein agar plate. Strains that produced clearance zones in this medium were selected as protease producers.

Crude enzyme preparation

The selected cultures were then were grown in FMB (Feather meal broth) consisting of (g l^{-1}): (10, feather meal; 0.5, NaCl; 0.3, K_2HPO4; 0.4, KH_2PO_4; 0.1, $MgCl\text{-}6H_2O$; 0.1, yeast extract). About 5% of 16 h old culture was used to inoculate the flasks containing 100 ml of FMB medium which were incubated at 30°C for 96 h with agitation for enzyme

production. The culture was centrifuged at 4ºC in order to harvest clear supernatant containing keratinase[8].

Enzyme assay

The above said crude enzyme preparations were used to determine enzyme activity in order to select the isolate producing maximum enzyme activity. Keratinolytic activity was determined by the method as described by Shrinivas *et al* (2011)[8] using feather meal powder as substrates. One Unit of keratinolytic activity was defined as the amount of enzyme required to release 1 µg of tyrosine per ml under experimental condition.

Identification of the selected fungal strains

The selected fungal strains were identified based on morphological and sporulation studies.

Protein estimation

Protein content of the crude enzyme was estimated by the method described by Lowry *et al* (1951)[9] using BSA as standard.

Characterization of keratinase

Time course of keratinase production

The enzyme production by the selected fungal strains was determined at different period of time for deducing the optimal production time.

Effects of pH on enzyme activity and stability

The pH optimum of the enzyme activity was studied by assaying the crude enzyme extracts at various pH (7 -10). The pH stability was determined by incubating keratinase in buffers of varying pH (7 -10) at 30°C for 60 min and the residual activity was determined.

Effects of temperature on enzyme activity and stability

The optimum temperature of the enzyme was measured by incubating the crude enzyme extracts at temperatures ranging from 30 to 90^{o}C, pH 9.0 and determining enzyme activity. Thermal stability was assessed by incubating the enzyme at 30 to 90^{o}C for 60 min and calculating the residual activity.

Degradation of keratin wastes

The capacity of degradation of keratin substrates was tested by inoculating the fungal strains on FMB medium containing 1% intact raw feathers at 30 ^{0}C for a period of 96 h. Degradation of substrate was visually inspected.

RESULTS AND DISCUSSIONS

All the experiments were conducted in triplicates and the results given are mean ± standard error.

Isolation screening and identification of keratinolytic fungal strains

In the current study, nine fungal strains were isolated from the soil samples collected from poultry waste dumping site, Goa by baiting technique (Fig 1). All the isolates were screened for enzyme activity on the casein agar plates.

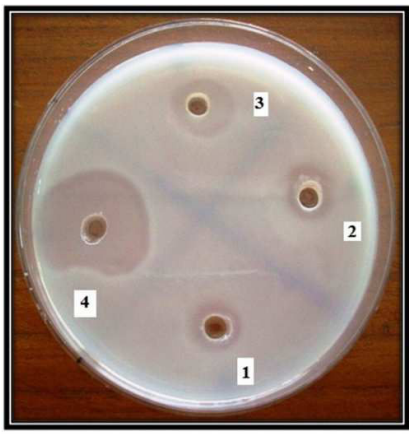
Figure 1
Hair baiting technique

Sterile defatted feather wastes on petri plate containing soil sample

Fungal growth after 21 days

The organisms producing zone of hydrolysis on casein agar plates were considered as proteolytic organisms. Among all nine strains, 4 isolates showed positive on casein agar plate (Fig 2).

Figure 2
Clearance on casein agar plate by the crude enzyme extracts



Enzyme assay for the four isolates revealed that the strains designated as 3 and 4 exhibited highest keratinase activity of 150 U/ml and 90 U/ml and were identified as C*hrysoporium sp and Microsporum sp.* respectively (Table 1).

Table 1
Quantitative estimation of keratinase activity

Isolate	Enzyme Activity (U/ ml)
1	35
2	40
3	150
4	90

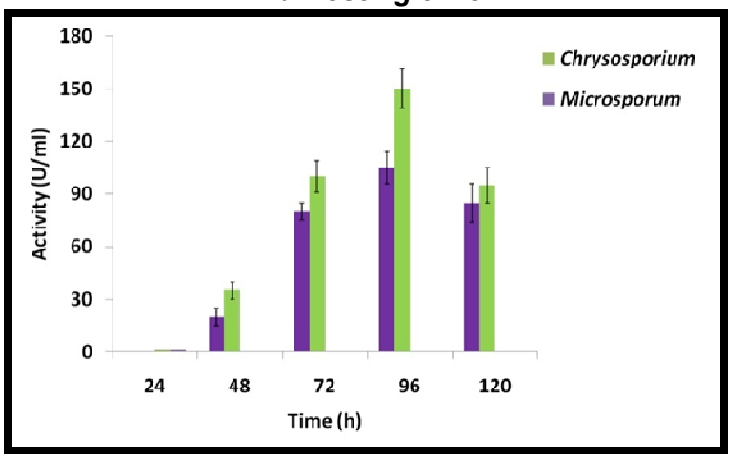
In natural environments, keratinolytic fungi are involved in recycling the carbon, nitrogen, and sulfur of the keratins. Their presence and distribution seem to depend on keratin availability. A number of studies focused on the keratinolytic potential of dermatophytic fungi are available. Little commercial interest was attracted by this group because of their potential pathogenicity. Besides the biotechnological interest, these investigations may help in understanding the role of fungi in the degradation of complex keratinous substrates in the nature[5].

Characterization of the keratinase enzymes

Harvesting time

The result of the experiment (Fig. 3) revealed harvesting time of 96 h was ideal for maximum enzyme production by both strains. The keratinolytic activity increased with the increase of time till 96 h after which there was decline in the enzyme production. This could be due to catabolite repression mechanisms. The rate of increase of the enzyme activity is not similar in pattern for all the isolates. Jahan *et. al,* (2010)[10] reported that highest enzyme production by *Bacillus* sp. after 72 h of cultivation on feather meal.

Figure 3
Harvesting time



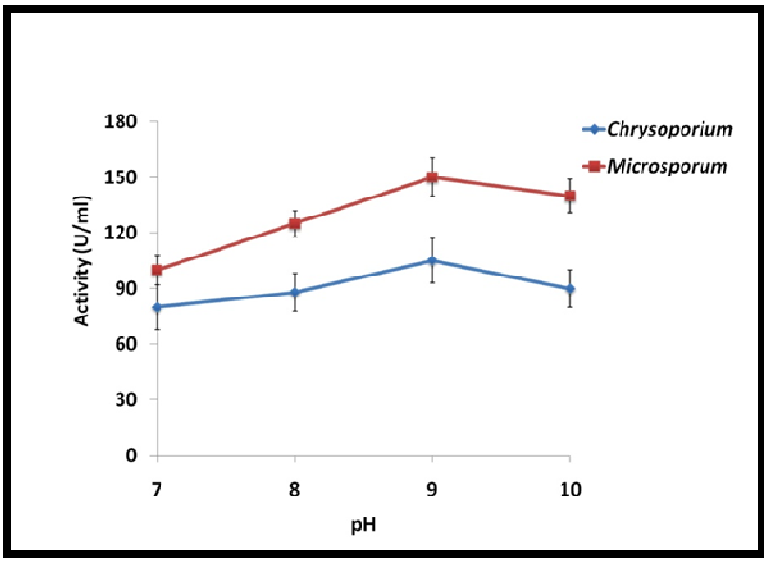
Growth conditions: pH 7.0, 30°C, 120 h under agitation

Keratinase production is highly variable, depending on the microorganism, the substrate and the carbon and nitrogen concentration, implicating that the medium composition should be determined on a case-by-case basis[11]. Keratin degradation takes from 24 h to several days. This is probably attributed to the complex mechanism of keratinolysis of these microorganisms[12].

Effects of pH on enzyme activity and stability

The effect of pH on the activity of keratinase in the range of 7.0 -10.0 showed a gradual increase in keratinase activity with increase in pH from 7.0 with maximum activity at pH 9.0 (Fig 4).

Figure 4
pH optima



Assay condition: pH 7 -10, 30°C, 30 min

An overview of literature on pH and temperature stability indicates that keratinases are generally active and stable over a wide range of pH from 5 to 13. Keratinases from most bacteria, actinomycetes and fungi have pH optima in neutral to alkaline range. However, a few keratinases possess extreme alkalophilic optima of pH>12 [13]. Enzyme with optimum activity at alkaline pH has definite advantage in application both in degradation of feather as well as in leather industry as significant increase in pH are found associated in the processes. The present enzymes were found to be stable between pH 7.0 and pH 9.0 for a tested period of 60 min by retaining more than 80 % of the initial activity (Fig 5 a & b). This pH stability confers it a potential to be used in various industrial sectors.

Figure 5a
pH stability of the enzyme extract from *Chrysosporium sp*

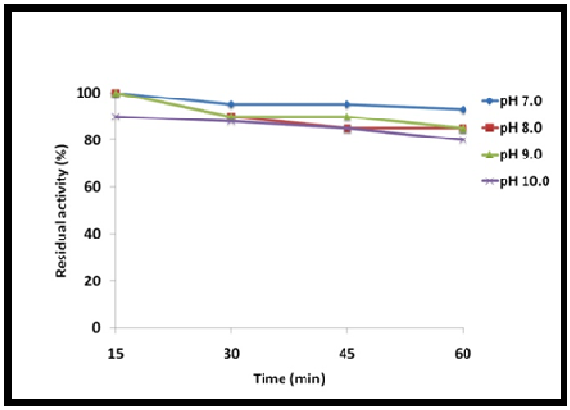
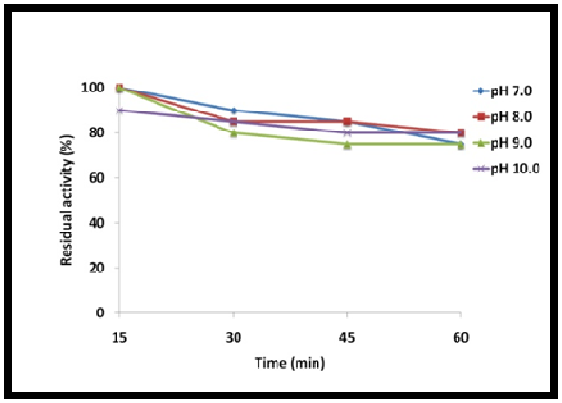


Figure 5b
pH stability of the enzyme extract from *Microsporum sp*

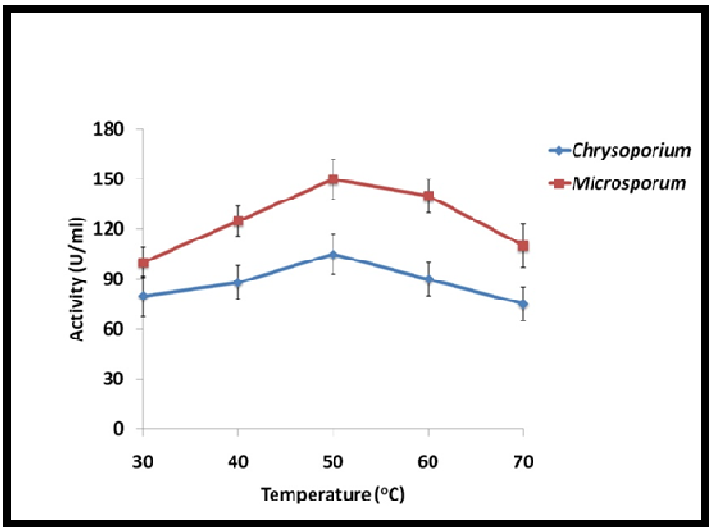


Assay condition: pH 7 -10, 30°C, 60 min

Effects of temperature on enzyme activity and stability

The enzymes showed optimal activity at 50 ^{0}C indicating thermo-tolerant nature of the enzyme (Fig 6). Further increase in temperature resulted in decline in the enzyme activity. The Fig 7 a & b shows thermal stability of the enzymes at 30-70 ^{0}C indicating the retention of at least 55% activity for a time period of 60 min heat treatment.

Figure 6
Temperature optima



Assay condition: pH 9, 30°- 60° C, 30 min

Figure 7a Temperature stability of the enzyme enzyme extract from *Chrysosporium sp*

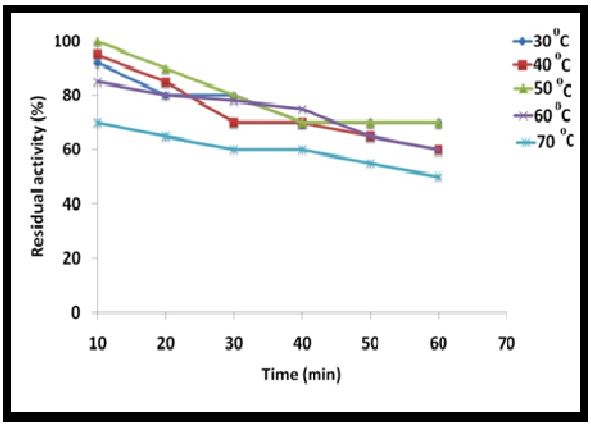
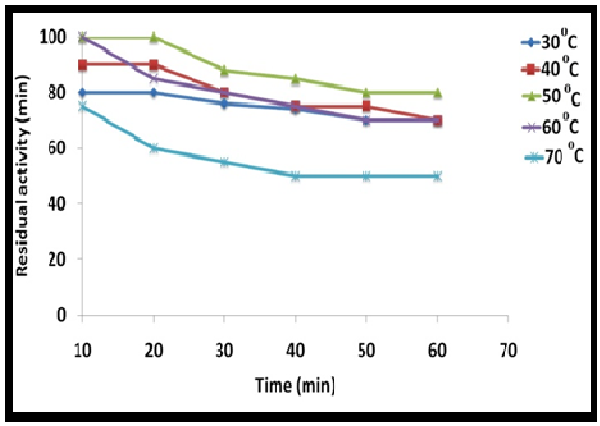


Figure 7b Temperature stability of the extract from *Microsporum sp*



Assay condition: 30°- 60° C, pH 9.0, 60 min

Optimum temperature of activity from majority of the mesophilic microorganisms producing keratinase has been observed to be between 28-45°C [14, 4]. An optimum keratin-degrading activity at mesophilic temperatures should be a desirable characteristic because these microorganisms may achieve hydrolysis with reduced energy input[15].

The temperature optima of keratinases may also be very variable, often depending on the source and origin of the isolate. The enzyme of the thermophilic *F. pennavorans* has

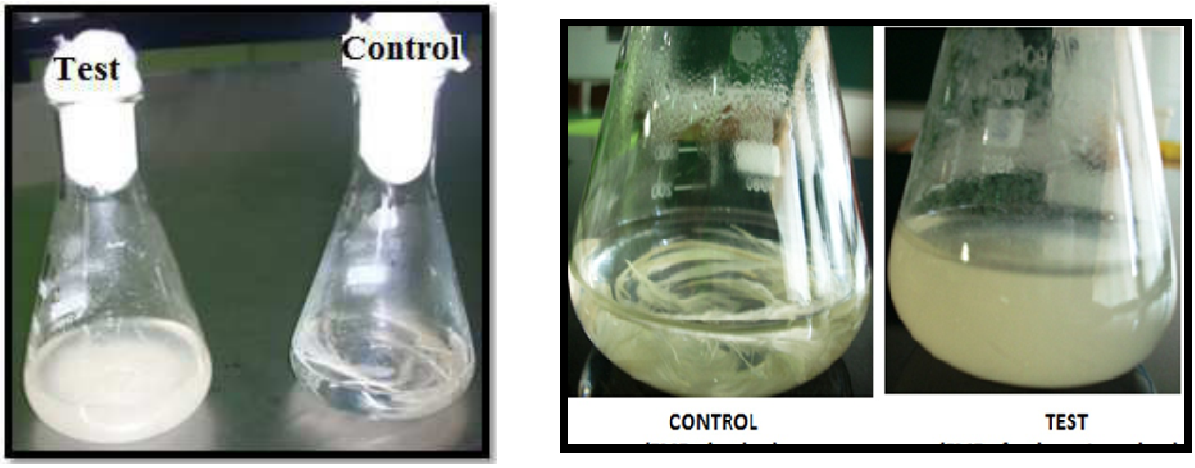
optimum temperature at 80°C while the mesophilic *Stenotrophomonas maltophila* DHHJ showed maximum activity at 40°C[5].

Degradation of keratin wastes

Macroscopic digestion of feather by the fungal strains increased considerably upon prolonged incubation resulting in a color change from a roughly colorless medium to a yellowish fermentation broth. Whole decomposition of chicken feather was eventually observed within 4 days (Fig 8a & b). These results suggest that the keratinase produced by both fungal strains is capable of digesting chicken feathers.

Figure 8a Feather degradation by *Chrysosporium sp*

Figure 8b Feather degradation by *Microsporum sp*



Test: FMB medium containing 1% intact raw feathers inoculated with the respective fungal strain at 30 ^{0}C, 96 h under agitation. Control is without the fungal strain.

The complete mechanism of keratin degradation is not fully understood. Basically, microbial keratinolysis is a proteolytic, protein-degrading process for the simple reason that keratin. The high mechanical stability of keratin and its resistance to proteolytic degradation is due to the tight packing of the protein chains through intensive inter linkage by cystine bridges. The capability of filamentous fungi to degrade keratin may be the result of a combination of extracellular keratinase, mechanical keratinolysis (mycelial pressure and/or penetration of the keratinous substrate), sulphitolysis (reduction of disulphide bonds by sulphite excreted by mycelia) and proteolysis[13]. Enzymatic or chemical reducing agents in form of disulfide reductases, sulfite, thiosulfate or cellular membrane potential may play a significant role in the degradation of this insoluble protein, additionally, the initial attack by keratinases and disulfide reductases may allow other less specific proteases to act, resulting in an extensive keratin hydrolysis[16].

CONCLUSION

The practical use of keratinase producing microorganisms is being explored in applied microbiology where there is great need for active degraders of feather keratin[17]. Environmental pollution and degradation of ecosystem have assumed significance owing to an increase in the accumulation of wastes from industries, agriculture and poultry. In India, poultry feather animal hair and other keratin sources do not find suitable applications. Surveys conducted at different feather dumping soils in various places indicated that several tons of poultry feather go as waste every day[18].

Soils that are rich in keratinous materials are the most conducive for the growth and occurrence of keratinophilic fungi[19]. Keratinophilic fungi are important ecologically

and present in the environment with variable distribution patterns. If keratinophilic fungi were not there to cycle this highly stable protein (keratin), then one can imagine the quantity of keratin that would have accumulated on earth, since a vast quantity of keratin is shed by the vertebrates. Indian soils contain many more keratinophilic fungi than those presently recorded, and there is need for further taxonomic and ecological studies of this interesting group of organisms [6]. The keratinolytic fungus isolated in this study could play an important role in production of animal feed protein in addition to the biodegradation of poultry wastes for betterment of environmental hazards. With this unique property, these keratinases are likely to occupy a special niche among proteases. The present century can look forward to these enzymes for addressing challenging issues of solid waste management. Considering the overall results of this present investigation, the reported keratinases could be an alternative enzyme/s for the industrial applications of the degradation of feather keratin to overcome the problem of environmental contamination with poultry wastes, but surely this will demand further testing and evaluation. With this unique property, these keratinases are likely to occupy a special niche among proteases. The present century can look forward to these enzymes for addressing challenging issues of solid waste management.

ACKNOWLEDGMENT

The financial support for this research work is provided by University Grants Commission, India (file no: 47-1645/10 (WRO).

REFERENCES

1. Tapia Daniel MT and Contiero J, Production and partial characterization of keratinase produced by a microorganism isolated from poultry processing plant waste water. Afr J Biotechnol, 7(3):296-300, (2008).
2. Joshi GS, Tejashwini MM, Revati N, Sridevi R, and Roma D, Isolation, Identification and Characterization of a feather degrading Bacterium. Int J Poultry Sci, 6(9): 689-693, (2007).
3. Brandelli A and Riffel A, Production of an extracellular keratinase from *Chryseobacterium sp.* growing on raw feathers. Electronic J Biotechnol, 8(1):35-42, (2005).
4. Ramnani P, Singh R and Gupta R, Keratinolytic potential of *Bacillus licheniformis* RG1: structural and biochemical mechanism of feather degradation. Can J Microbiol 51:191–196, (2005)
5. Brandelli A, Daroit DJ and Alessandro Riffel A, Biochemical features of microbial keratinases and their production and applications. Appl Microbiol Biotechnol 85:1735–1750, (2010).
6. Sharma R and Rajak RC, Keratinophilic Fungi: Nature's Keratin Degrading Machines- Their Isolation, Identification and Ecological Role. Resonance, 8: 28-40, (2003).
7. Agrahari S and Wadhwa N, Degradation of chicken feather, a poultry waste product by keratinolytic bacteria isolated from dumping site at Ghazipur poultry processing plant. Int J Poult Sci, 9:(5) 482-489, (2010).
8. Shrinivas D, Kumar R and Naik GR, Enhanced production of alkaline thermostable keratinolytic protease from calcium alginate immobilized cells of thermo alkalophilic *Bacillus halodurans* JB 99 exhibiting dehairing activity. J Ind Microbiol Biotechnol, 39 (1):93-98, (2011).
9. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. J Biol Chem, 193: 265-275, (1951).
10. Jahan Z, Khan SN, and Mozammel Hoq M, Screening of keratinolytic bacteria

from Poultry Wastes. Bangladesh J Sci Ind Res, 45(3):261-266, (2010).
11. Cai C and Zheng X, Medium optimization for keratinase production in hair substrate by a new *Bacillus subtilis* KD-N2 using response surface methodology. J Ind Microbiol Biotechnol, 36:(7) 875-883, (2009).
12. Ramnani, P and Gupta R. Optimization of medium composition for keratinase production on feather by *Bacillus licheniformis* RG-1 using statistical methods involving response surface methodology. Biotechnol Appl Biochem, 40:491–496, (2004).
13. Gupta R and Ramnani P, Microbiol keratinases and their prospective applications: An overview. Appl Microbiol Biotechnol, 70 (1):21-33, (2006).
14. Allpress JD, Mountain G and Gowland PC, Production, purification and characterization of an extracellular keratinase from Lysobacter NCIMB 9497. Lett Appl Microbiol, 34:337-342, (2002).
15. Tamilmani P, Umamaheshwari A, Vinayagam A and Prakash B, Production of an extracellular feather degrading enzyme by *Bacillus licheniformis* isolated from poultry farm soil in Namakkal district (Tamilnadu). Int J poultry sci, 7(2):184-188, (2008).
16. Gioppo N MR, Gasparin FGM, Costa AM, Alexandrino AM, DeSouza CGM and Peralta RM, Influence of the carbon and nitrogen sources on keratinase production by *Myrothecium verrucaria* in submerged and solid state cultures. J Ind Microbiol Biotechnol, 36:705-711, (2009).
17. Deivasigamani B and Alagappan KM, Industrial application of keratinase and soluble proteins from feather keratins. J Environ Biol, 29(6): 933-936, (2008).
18. Tawfik M, Mushin and Rawa B Hadi, Degradation of keratin substrates by fungi isolated from sewage sludge. Mycopathologia, 154:185-189, (2001).
19. Moallaaei H, Zaini F, Larcher G,Beucher B and Bouchara JP, Partial purification of a 37 Kda extrecellular proteinase from *Trichophyton vanbreuseghemii.* Mycopathologia 161:369-375, (2006).