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Native Feather Degradation by a Keratinophilic Fungus

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Abstract: A novel feather-degrading fungus belonging to *Aspergillus* genus was isolated from poultry waste in Goa, India. Culture medium was optimized for the production of maximum amount of extra cellular keratinase in an inexpensive feather meal broth medium using chicken feather as a sole of carbon and nitrogen source resulting in 43 fold increase in enzyme production. The enzyme was characterized for its optimal pH, temperature, pH stability and temperature stability over a wide range. The strain was able to completely degrade native feather within a period of 96 h. These results suggest potential biotechnological applications of this *Aspergillus* strain that involve hydrolysis of keratin, including the improvement of the nutritional properties of feathers (and other keratins) used as supplementary feedstuffs.
Key Words: Feather, feather degradation, keratinase, keratinolytic activity, poultry wastes.

Introduction:

Feather wastes are generated in large quantities as a by product of commercial poultry processing as well as through natural processes. Feathers are made up primarily of keratin which is resistance to common proteolytic enzyme such as pepsin, trypsin and papain [1]. 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 [2]. Till date most of purified keratinizes known cannot completely solubilize native keratin, their exact nature and uniqueness for keratinolysis is still not clear [3]. There is a need 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 [4]. In view of above, the present study was aimed to optimize the growth conditions of a potent keratinophilic microbial strain for maximum enzyme production and its efficacy to degrade keratin rich substrates.

Materials and Methods:

Isolation of Keratinophilic Fungi

The soil samples were collected from the feather processing areas, Goa, India. Hair baiting technique was followed for the isolation of keratinophilic fungi [5]. 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 [4]. 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.

Inoculum preparation and Enzyme production

The strains grown by baiting technique were inoculated on casein agar plates and those produced clearance zones in this medium considered as proteolytic strains were selected for subsequent quantitative screening for the final selection of true keratinophilic fungal strain/s. The selected proteolytic fungal cultures were grown in 100 mL of Feather meal broth (FMB) consisting of (g l^{-1}): (10, feather meal; 0.5, NaCl; 0.3, K_2HPO_4; 0.4, KH_2PO_4; 0.1, MgCl-$6H_2O$; 0.1, yeast extract). About 5% of 16 h old culture was used to inoculate the flasks which were incubated at 30°C for 24 h for enzyme production. The culture was centrifuged at 4ºC to harvest clear supernatant (crude enzyme extract) containing keratinase. Keratinolytic activity of the extract was determined by the method as described by Shrinivas and Naik [6]. 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 strain was 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* [7] using BSA as standard.

Optimization of cultural parameters for keratinase production

The effect of agitation on enzyme production was studied by growing the isolate in FMB medium under static/shake flask condition and the enzyme production was monitored after 24 h. The enzyme production was determined at different period of time for deducing the optimal production time. The influence of temperature and pH on production of keratinase was studied at 27ºC, 37ºC and 45ºC ($\pm$2 ºC) and pH 6.0 - 9.0. The effect of varying amounts of substrate (feather meal powder, 0% - 2.0%) and yeast extract (0.02% - 0.08%) on enzyme production were investigated.

Characterization of keratinase

The pH and the temperature optima of the enzyme activity was studied by assaying the crude enzyme extract at various pH (6 -10) or at various temperatures (37, 45, 50 to 55^{o}C, pH 9.0) respectively. The pH stability was determined by calculating the residual activity of the enzyme after incubating the enzyme in the buffer of optimum pH at 28 $\pm$ 2°C for 60 min. Likewise thermal stability was assessed by incubating the enzyme at optimum temperature 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 strain on FMB medium containing 1% intact raw feathers at 30°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 strain

In the current study, nine fungal strains were isolated from the soil samples collected from poultry waste dumping site, Goa, India by baiting technique (Fig 1 a and b). All the isolates were screened for enzyme activity on the casein agar plates. The organisms producing zone of hydrolysis on casein agar plates were considered as proteolytic organisms. Among all nine strains, four isolates showed positive on casein agar plate. Keratinase assay for the four isolates revealed that the strain designated as FK1 exhibited highest enzyme activity of 3.5 U/ml (Table 1) and identified as *Aspergillus* sp.

Fig 1 a Sterile defatted feather wastes on petri plate containing soil sample

Fig 1 b Fungal growth after 21 days

Figure 1 Hair baiting technique

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].

Optimization of cultural parameters for keratinase production

The culture grown under agitation yielded maximum enzyme production (Fig. 2). Keratinase has been produced under submerged shaking conditions, except for a few thermophilic bacteria where static submerged fermentation has been reported [8]. During the fermentation, different levels of dissolved oxygen in the fermentation broth can be obtained by variations in the agitation speed. This can influence greatly cell growth of the microbes and thereby production of extracellular enzymes [9].

The harvesting time of 72 h was found to be ideal for maximum enzyme production (Fig. 3). Similar results were reported by Jahan *et al* [8] and Kanchana [10] for enzyme production by *Bacillus* sp. cultivated on feather meal. Keratin degradation takes from 24 h to several days. This is probably attributed to the complex mechanism of keratinolysis of these microorganisms [9].

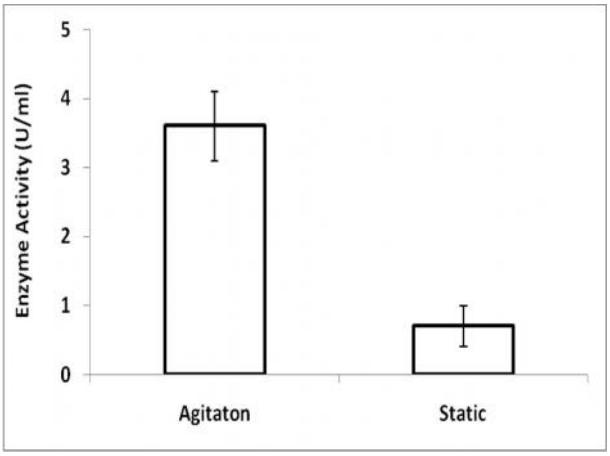

Fig 2 Effect of agitation vs static condition

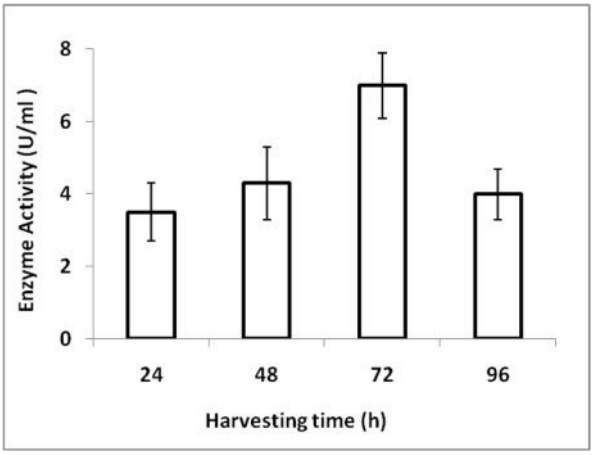

Fig 3 Harvesting time:

The temperature of 37°C yielded the maximum enzyme production (Fig. 4). These results are similar to that reported for keratinases of other microorganisms, such as *Trichophyton* sp [1]. Elevated temperature might favour increased cell growth, but not enzyme production. The pH 7 favoured the maximum enzyme production (Fig. 5). The pH of the medium is a significant factor that influences the physiology of a microorganism. It has been observed that pH from 7 to 9 supports keratinase production and feather degradation in most microorganisms [11]. Alkaline pH possibly favours keratin degradation as higher pH modifies cystine residues making it accessible for keratinase action.

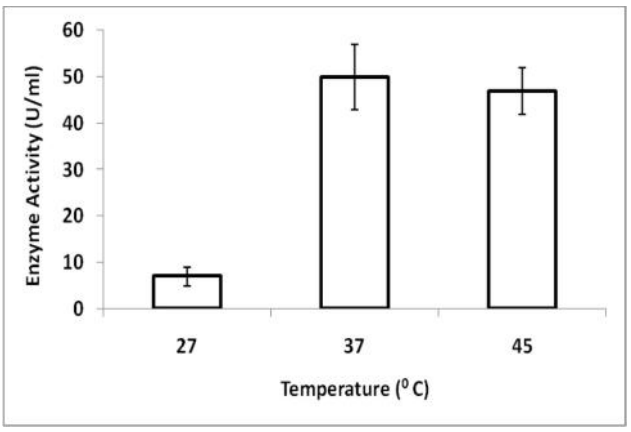

Fig. 4 Effect of temperature

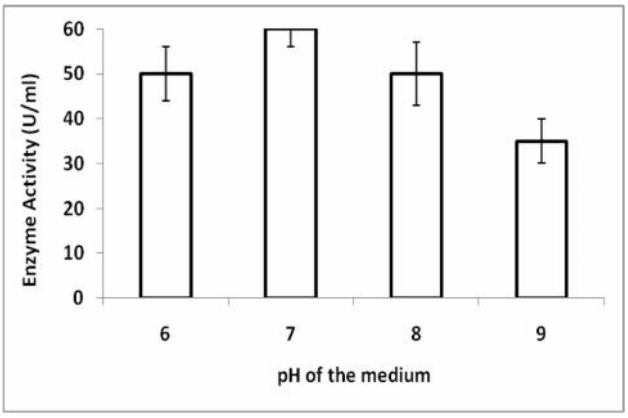

Fig.5 Effect of pH

The optimum concentration of feather meal was found to be 1.5 % for enzyme production (Fig. 6). Inhibition of keratinase production after 1.5 % of feather meal as observed in the present study indicating that this strain has a catabolite repression regulatory mechanism, a common controlled mechanism for biosynthesis of bacterial proteases including keratinases. Most of the reports available on keratinases, group them as inducible enzymes; however, few constitutive keratinases have also been reported [12]. It is important to mention that in most of the reports on constitutive keratinases, the nature of the enzyme is based on their caseinolytic rather than keratinolytic activity. Hence, it is proposed that keratinolytic activity is by and large inducible [1].

The concentration of yeast extract for maximum enzyme production was found to be 0.06% (Fig 7). Feather medium supplemented with 0.1% (w/v) yeast extract as an external organic nitrogen source showed maximum production of keratinase by *Bacillus* sp. but enzyme production was decreased when the yeast extract concentration was increased to 1% (w/v) [13].

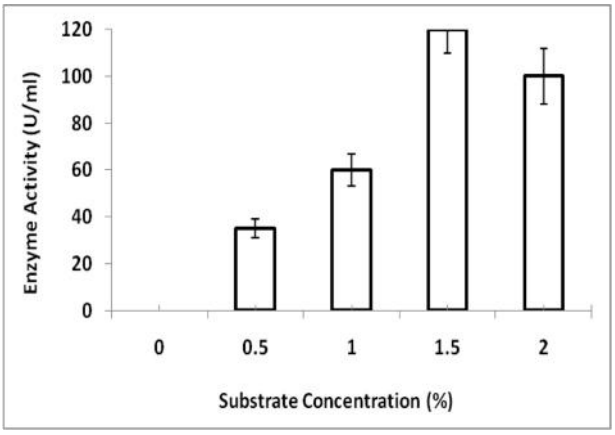

Fig.6 Effect of feather meal concentration

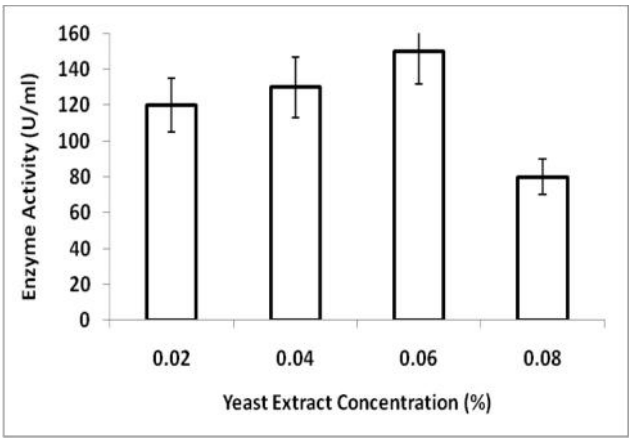

Fig.7 Effect of yeast extract concentration

The optimized medium greatly improved the keratinase production (approximately 43 fold increase). Additionally, this culture medium demonstrated high efficiency in the degradation of non-soluble feather to soluble protein which has potential uses in feed industry through the simple biotechnological treatment by *Aspergillus* sp.

Table 1 Quantitative estimation of keratinase activity

Isolate	Enzyme Activity (U/ ml)
FK 1	3.5
FK 2	3.0
FK 3	2.4
FK 4	2.2

FK: Fungal keratinase

Characterization of keratinase

The effect of pH on the activity of keratinase in the range of 6.0 -10.0 showed a gradual increase in keratinase activity with increase in pH from 7.0 with maximum activity at pH 9.0 (Fig 8). 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 [1]. 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 enzyme was found to be stable at pH 9.0 for a tested period of 60 min by retaining more than 80 % of the initial activity (Fig 9). This pH stability confers it a potential to be used in various industrial sectors.

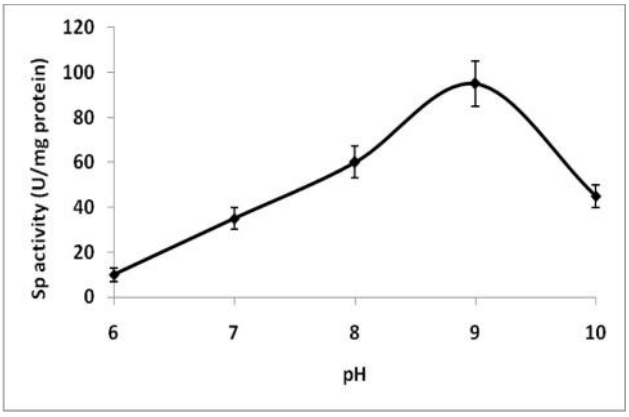

Fig. 8 Optimum pH

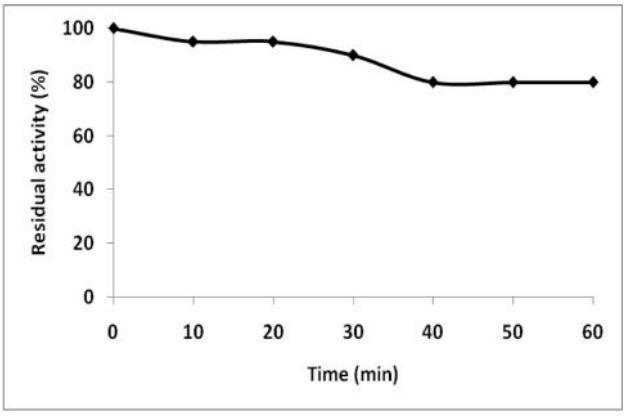

Fig. 9 pH stability

The enzyme showed optimal activity at 45^0C indicating thermo-tolerant nature of the enzyme (Fig 10). Further increase in temperature resulted in decline in the enzyme activity. The Fig 11 shows thermal stability of the enzyme at 45^0C indicating the retention of at least 60% activity for a time period of 60 min.

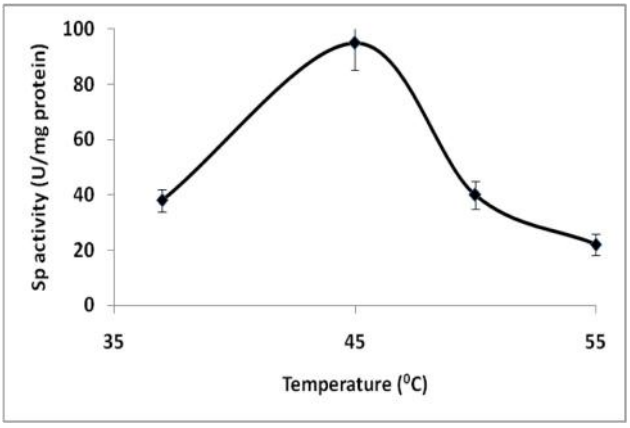

Fig 10 Optimum temperature

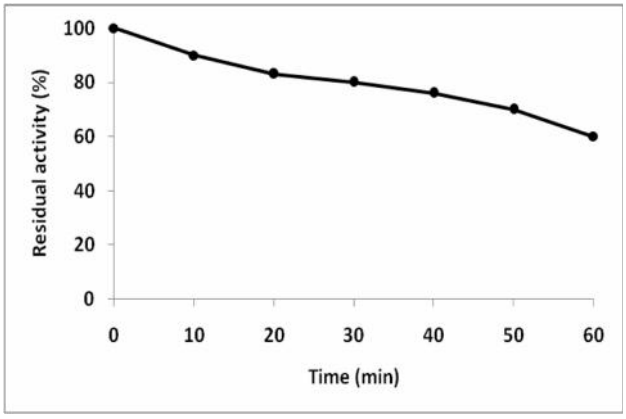

Fig 11 Thermal stability

Optimum temperature of activity from majority of the mesophilic microorganisms producing keratinase has been observed to be between 28-45°C [14, 3]. 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 *Fervidobacterium pennavoran* has optimum temperature at 80°C while the mesophilic *Stenotrophomonas maltophila* DHHJ showed maximum activity at 40°C [16].

Degradation of keratin wastes

Macroscopic digestion of feather by the fungal strains increased considerably upon prolonged incubation resulting in a colour change from a roughly colourless medium to a yellowish fermentation broth. Whole decomposition of chicken feather was eventually observed within 4 days (Fig 12). These results suggest that the keratinase produced by the fungal strain is capable of digesting chicken feathers. This result is in par with the report by Kanchana [17] where the feather degradation by C*hrysoporium sp and Microsporum sp.* under similar conditions were investigated.

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 is a protein. 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 [1]. 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 [18].

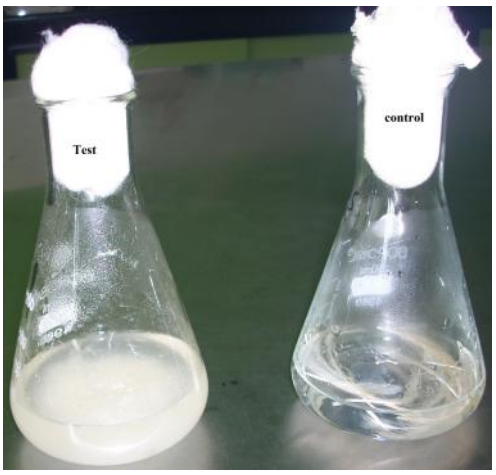

Fig. 12 Degradation of chicken feathers by *Aspergillus* sp

Control :(FMB + 1% intact raw feather), pH 7.0, 30°C, 96 h, agitation
Test: (FMB + inoculum + 1% intact raw feather), pH 7.0, 30°C, 96 h, agitation

Conclusion:

The production of keratinase from *Aspergillus* sp. is easy to scale up; the organism grows on simple media with feathers as its sole carbon, nitrogen, and energy source. Hence, it is possible to culture an organism with great commercial potential using an inexpensive substrate, resulting in low production cost. At the same time, it transforms a kind of industrial waste (chicken feather) into the required nutritional feed additives, thereby protecting the environment by minimizing wastage. Further studies should therefore be carried out in order to evaluate the biotechnological potential of this keratinase in processes involving keratin hydrolysis. The rapid growth of poultry industry has linked with increased output of keratin containing wastes. Keratinous wastes can be readily fermented to useful products and commodity chemicals by the appropriate microbes. The present research concerns about the biodegradation of keratinous wastes. The keratinolytic microorganisms isolated in this study therefore could play an important role in production of animal feed protein in addition to the biodegradation of poultry wastes. Finally to our knowledge this is the first report concerning on isolation of *Aspergillus* sp as keratinolytic fungi from soil samples in studied areas in Goa, India.

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References:

1. Gupta R. and Ramnani P., Microbial keratinases and their prospective applications: an overview, Appl Microbiol Biotechnol, 2006, 70, 21-33.
2. Brandelli A., Hydrolysis of native proteins by a keratinolytic strain of *Chryseobacterium* sp, Ann Microbiol, 2005, 55, 47-50.
3. Ramnani P., Singh R. and Gupta R.,Keratinolytic potential of *Bacillus licheniformis* RG1: structural and biochemical mechanism of feather degradation, Can J Microbiol, 2005, 51, 191-196.
4. 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 Poul Sci, 2010, 9 (5), 482-489.
5. Sharma R. and Rajak RC., Keratinophilic Fungi: Nature's keratin degrading machines- Their isolation, identification and ecological role, Resonance, 2003, 8, 28-40.
6. Shrinivas D. and Naik GR., Characterization of alkaline thermostable keratinolytic protease from thermoalkalophilic *Bacillus halodurans* JB 99 exhibiting dehairing activity, Int. Biodeterioation & Biodegradation, 2011, 65(1), 29-35.
7. Lowry O.H., Rosebrough N.J, Farr A., Randall R.J., Protein measurement with the Folin phenol reagent.,J Biol Chem, 1951, 193,265-275.
8. Jahan, Khan S.N., Mozammel Hoq M., Screening of Keratinolytic bacteria from poultry wastes, Bangladesh J Sci Ind Res , 2010,45(3), 261-266.
9. 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, 2004, 40,491-496.
10. Kanchana R., Farm waste recycling through microbial keratinases, J Appl Sci Environ Sanitation, 2012, 7 (2), 103-108.
11. Brandelli A. and Riffel A., Production of an extracellular keratinase from *Chryseobacterium* sp. growing on raw feathers, Electronic J Biotechnol, 2005, 8,35-42.
12. Manczinger L., Rozs M.C.V., Kevei F., Isolation and characterization of a new keratinolytic *Bacillus licheniformis* strain, World J Microbiol Biotechnol , 2003,19,35-39.
13. Kainoor P.S. and Naik G.R., Production and characterization of feather degrading keratinase from *Bacillus* sp. JB 99, I J Biotechnol, 2010, 9.384-390.
14. Allpress J.D., Mountain G. and Gowland P.C., Production, purification and characterization of an extracellular keratinase from *Lysobacter* NCIMB 9497,. Lett Appl Microbiol, 2002, 34,337-342.
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, 2008, 7(2), 184-188.
16. Brandelli A., Daroit D.J. and Alessandro Riffel A., Biochemical features of microbial keratinases and their production and applications, Appl Microbiol Biotechnol, 2010, 85,1735-1750.
17. Kanchana R., Utilization of biodegradable keratin containing wastes by enzymatic treatment.,Int J Pharm Bio Sci , 2013, 4 (1), 117 -126.
18. Gioppo N.M.R., Gasparin F.G.M., Costa A.M., Alexandrino A.M., DeSouza C.G.M., Peralta R.M., Influence of the carbon and nitrogen sources on keratinase production by *Myrothecium verrucaria* in submerged and solid state cultures, J Ind Microbiol Biotechnol, 2009, 36,705-711.

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