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Effect of Different Operational Parameters on Bio-degradation of Chicken Feathers by *Aspergillus niger*: Investigation Under Submerged Fermentation Process

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Abstract. Fungal strain, *Aspergillus niger* (ATCC 1015) has ability to grow on keratinous material therefore, it was selected for the investigating bio-degradation of chicken feathers. Different operational parameters were studied under submerged fermentation process i.e. effect of substrate concentration, effect of pH, effect of incubation temperature, effect of yeast extract concentration and effect of volume of fermentation medium. *A. niger* was grown on solid medium of malt extract and agar, due to its ability of rapid growth on it. Complete bio-degradation of the substrate was achieved after 5 days (0.70±0.03 U/mL) under standard optimized conditions. Investigation of different operational parameters on bio-degradation of chicken feathers revealed, maximum keratinolytic was observed at 40 °C incubation temperature, at 0.5 g/100 mL of substrate concentration, 8 g/100 mL concentration of yeast extract, at 7 pH of the fermentation medium and at 50 mL volume of fermentation medium. The present study suggests that *A. niger* could prove to be a potential candidate for production of keratinase and bio-degradation of chicken feathers.

Keywords: *Aspergillus niger*, keratinase, bio-degradation, submerged fermentation, chicken feathers, operational parameters

Introduction

In recent years, poultry industry has expanded a lot due to massive increase in world population, which in turn has enhanced the demand of poultry products. Subsequently problematic waste production from the poultry industry has been amplified too (Brandelli *et al.*, 2015; Williams *et al.*, 1991). The recent outbreak of bird flu caused by H5N1 virus was also due to waste feathers that pollute our environment (Saber *et al.*, 2009). The accumulated waste of chicken feathers creates potential environmental issues because of their resistant nature (Anitha and Palanivelu, 2013).

The bio-technological bio-degradation of agro-industrial waste, especially chicken feathers have numerous advantages including, cost effectiveness, preservation of essential amino acids, utilization for animal feed and fertilizer production as well as it's an environmentally friendly approach. Instead of chemical processing which produces several harmful waste effluents, loss of nutrients, less useful for further utilization of by products (Mazotto *et al.*, 2013; Deivasigamani and Alagappan, 2008; Riffel *et al.*, 2003; Bressollier *et al.*, 1999).

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Feathers are mainly composed of keratin, which is a highly stable protein. Keratinous material is water insoluble and extremely resistant to degradation (Matikeviciene *et al.*, 2009). Keratinase enzyme is able to hydrolyse keratin and release the free amino acids from keratinous proteins (Shih, 1993). Fungi can readily degrade keratinous substances like chicken feathers by secreting large amount of extracellular keratinase into the culture medium (Anbu *et al.*, 2008). *Aspergillus* spp. are identified as keratinolytic fungi through number of researches done till present (Brandelli *et al.*, 2015; Saber *et al.*, 2009; Kim, 2003).

The objective of the present study was to investigate the production of keratinase enzyme and bio-degradation of the chicken feathers in submerged fermentation process, by a keratinolytic strain of *A. niger* (ATCC 1015). Data was processed in MS Excel 2013™ to make box plots and statistical analysis of different treatment units. A one way analysis of variance (ANOVA) and F distribution ($p \leq 0.05$) was conducted to compare results.

Materials and Methods

Preparation of substrate. Chicken feathers were collected from local poultry waste site and used as

substrate for production of keratinase for bio-degradation through *A. niger*. Attached meat and flesh were removed from the feathers and washed thoroughly, then dried in hot air oven at 50 °C for 30 min and were cut into tiny fragments. The fragments were sterilized for further experimentation.

Preparation of inoculum. The fungal strain of *A. niger* (Strain ATCC 1015), was taken from the Institute of Industrial Biotechnology, G.C. University Lahore.

The strains were cultivated on solid medium consisting of agar and malt extract (2 g/100 mL each). The slant cultures were incubated at 28 °C for 96-120 h until maximum growth was achieved. Sub-culturing was carried out after every 2 weeks and frequently examined under a light microscope to avoid any contamination.

For the preparation of 50 mL of inoculum, 40 mL of distilled water was taken in 250 mL Erlenmeyer flask containing chicken feathers (0.2 g/50 mL), yeast extract and peptone (1 g/50 mL each), $ZnSO_4$, $MgSO_4$ and $FeSO_4$ (0.05 g/100 mL each) and autoclaved at 121 °C, 15 lb/inch^2 for 15 min. 10 mL of sterilized distilled water was added in the slant and spores were suspended in the solution. The pH of the medium was maintained at 7, with the help of 1N HCl and 1N NaOH solutions. The flask was kept in a shaking incubator rotating at 120 rpm at 28 °C for 96 h.

Submerged fermentation. The fermentation medium was prepared by adding chicken feathers (0.2 g) along with yeast extract and peptone (2 g/100 mL each), K_2HPO_4 (0.2 g/100 mL) and $ZnSO_4$, $MgSO_4$, $FeSO_4$ (0.1 g/100 mL each) and were dissolved in 90 mL distilled water and then sterilized, then 10 mL of the inoculum was added in the flask and the pH of the medium was maintained at 7, with the help of 1N HCl and NaOH solutions. The flask was then kept in the shaking incubator rotating at 120 rpm at the temperature of 28 °C for 120 h. Since the enzyme is extracellular, it was extracted by centrifugation of the mixture followed by weighing the cell biomass.

Keratinolytic assay. Crude enzyme extracted as described in section 2.3, was used to evaluate enzyme activity. Protein content was determined using modified method of Mazotto *et al.* (2013) using tyrosine as standard. One Unit of keratinolytic activity was defined as the amount of enzyme required to release 1 µg of tyrosine per mL under experimental condition.

Keratinase activity was determined according to the modified Anson's method (Anson, 1938). 1.0 mL of the culture broth was taken in a 100 mL flask and 1.0 mL of pH 7.0 phosphate buffer was added in it. 1 mL of the substrate (2% tyrosine pH 7.0) was added in the buffer enzyme solution and incubated at 37 °C for 10 min in a water bath, then 10 mL of 5N TCA (Tri-chloro acetic acid) was added to stop the reaction. The precipitated keratin was then filtered off and 5 mL of the filtrate was taken in a test tube. Then 10.0 mL of 0.5N Na_2CO_3 solution and 3.0 mL of the Folin-Ciocalteu reagent were added in the filtrate. Final readings were taken with spectrophotometer at 550 nm. Blanks of the samples were prepared by adding the TCA before the addition of substrate.

Effect of different operational parameters on keratinolytic activity. ***Effect of different concentrations of substrate.*** For evaluating the effect of different concentrations of substrate on keratinolytic activity, concentrations ranging from 0.2-0.7 g/100 mL of fermentation medium were used for the biodegradation by *A. niger*. The pH of the medium was maintained at 7.0 and incubated at 28 °C for 96 h.

Effect of different pH. For investigating the effect of different pH on the bio-degradation of chicken feathers by *A. niger*, the fermentation medium was exposed to various pH at 5, 6, 7 and 8. The medium was incubated at 28 °C for 96 h and using 0.5 g chicken feathers as substrate in each flask.

Effect of incubation temperature. To determine the effect of different incubation temperatures on the bio-degradation of chicken feathers by *A. niger*, the fermentation medium was incubated at various temperatures i.e. 20, 30, 40 and 50 °C. The medium was incubated at 7 pH for 96 h and using 0.5 g chicken feathers.

Effect of different concentrations of yeast extract. To study the effect of different concentrations of yeast extracts on the bio-degradation of chicken feathers by *A. niger*, different concentrations of yeast extract (2 g, 4 g, 6 g and 8 g/100 mL) were added in the fermentation medium. The medium was incubated at 28 °C for 96 h, using 0.5 g chicken feathers and pH was maintained at 7.

Effect of different volumes of fermentation media. For the evaluation of the effect of fermentation volume on enzyme production, various volumes of fermentation media were prepared ranging from; 50 mL, 100 mL,

150 mL and 200 mL, the pH was maintained at 7 and 0.5 g of chicken feathers were added as substrate in each flask. The media were incubated at 28 °C for 96 h for the determination of its effect on biodegradation of chicken feathers.

Statistical analysis. All the experiments were performed in triplicates and results were expressed as mean ± Standard deviation. The data was statistically analysed by ANOVA (analysis of variance) considering $p<0.05$ as significant difference. Data was processed in MS Excel 2013™ to make box plots and statistical analysis of different treatment units. A one-way analysis of variance (ANOVA) and F distribution ($p\leq0.05$) was conducted to compare results.

Results and Discussion

Keratinolytic activity. This study was performed to evaluate the bio-degradation of chicken feathers and production of keratinase by *A. niger* under submerged fermentation process and to evaluate optimum levels pf different operational parameters for keratinolytic activity. The strain (ATCC 1015) of *A. niger* was able to successfully bio-degraded chicken feathers by submerged fermentation process as sole source of carbon. Keratinase enzyme was produced and chicken feathers were successfully bio-degraded within five days of submerged fermentation process (Table 1). It was previously observed that *A. niger* is a keratinolytic fungus and has the ability to produce extracellular enzyme kertinase.

Table 1. Bio-degradation of Chicken feathers by Aspergillus niger

Substrate concentration (g)	Specific (U/mL)			Mean value	Weight of cell biomass after fermentation (g)
0.50	0.60	0.72	0.79	0.68	0.39

The present work showed that 0.5 g/100 mL concentration of substrate, pH 7 of the fermentation medium, 50 mL volume of fermentation medium, 40 °C incubation temperature and 8 g/100 mL concentration of yeast extract were optimal conditions for the maximum biodegradation of chicken feathers and production of enzyme by *A. niger,* elaborated below.

Effect of different operational parameters on keratinolytic activity. ***Effect of different concentrations of substrate.*** *A. niger* gave highest production of enzyme (0.68±0.05 U/mL) at 0.5 g of chicken feathers, while lowest production of enzyme (0.30±0.03 U/mL) was at 0.7 g of chicken feathers (Fig. 1).

Effect of different pH. *A. niger* gave highest production of enzyme (0.60±0.03 U/mL) at pH 7, while lowest production of enzyme (0.40±0.02 U/mL) was at pH 8 (Fig. 2).

Effect of incubation temperature. *A. niger* gave highest production of enzyme (0.73±0.003 U/mL) at 40 °C, while lowest production of enzyme (0.65±0.02 U/mL) was at 50 °C (Fig. 3).

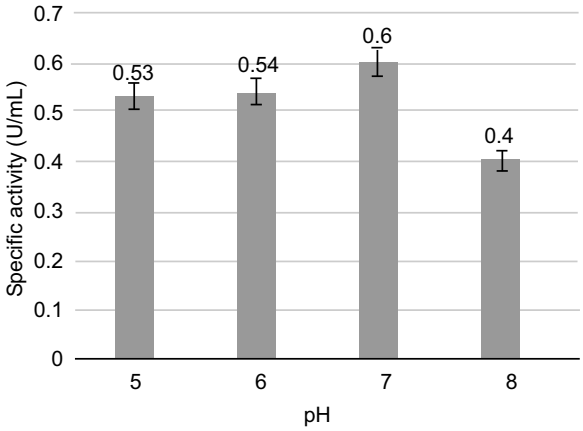

Fig. 1. Effect of substrate concentration on keratinolytic activity.

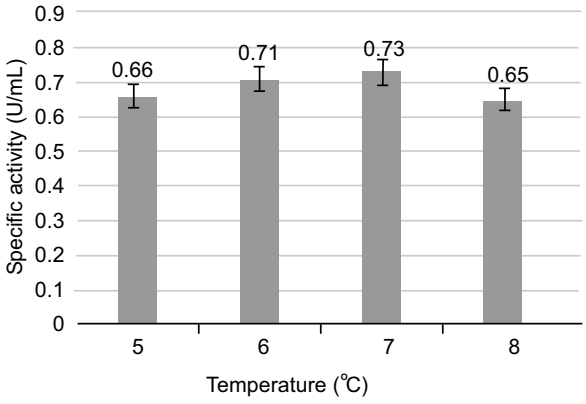

Fig. 2. Effect of pH on keratinolytic activity.

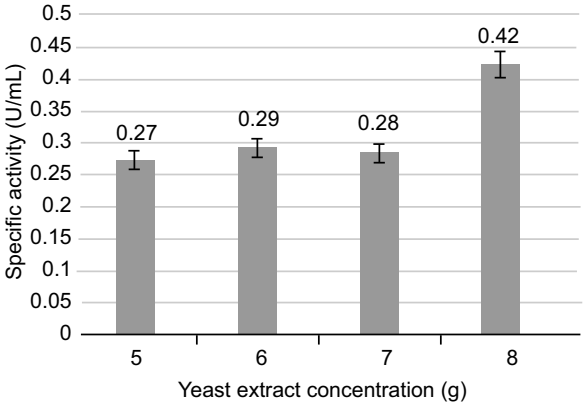

Fig. 3. Effect of temperature on keratinolytic activity.

Effect of different concentrations of yeast extract. *A. niger* gave highest production of enzyme (0.42±0.01 U/mL) at 8 g of yeast extract, while lowest production of enzyme (0.27±0.05 U/mL) was at 4 g of yeast extract (Fig. 4).

Effect of different volumes of fermentation media. The optimum conditions fermentation volume was 50 mL of fermentation medium, where highest production of enzyme (0.42±0.01 U/mL) was observed, while lowest production of enzyme (0.27±0.05 U/mL) was found at 200 mL of fermentation medium volume (Fig. 5).

The one-way analysis of variance (ANOVA) T-Test compared the measure of effect of different concentrations of substrate, pH, incubation temperature, yeast extract and fermentation volume on keratinolytic activity, the results were found statistically significant with F=15.2, F=6.2, F=3.5, F3.6 and 6.0 at a level of p=0.05, respectively.

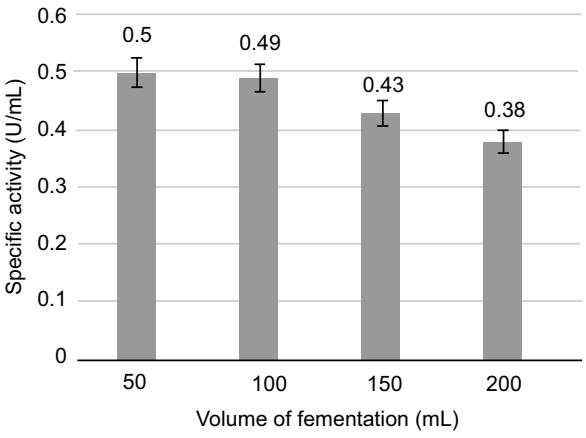

Fig. 4. Effect of yeast extract concentration on keratinolytic activity.

In this study, we aimed to observe the bio-degradation potential of *A. niger* and production of keratinase, in addition the bio-degradation was subjected to different operational parameters to identify the optimum conditions for maximum bio-degradation of substrate and production of enzyme. We found that *A. niger* strain ATCC 1015 partially degraded the substrate during growth in submerged fermentation medium with feather as the only carbon source. Similar results were described by Mazotto *et al.* (2013), they isolated 28 mutant species of *A. niger* from poultry waste site, and conducted bio-degradation of chicken feathers by submerged as well as solid state fermentation techniques. However, their study showed maximum keratinolytic activity in solid state fermentation as compared to submerged fermentation process. They reported the production of high mass peptidases and their results depict that *A. niger* is a promising candidate for bio-degradation of chicken feathers and production of keratinolytic enzymes. Other studies have also showed that solid state fermentation by *Aspergillus* spp. and bacterial species produce better results and have ability to produce more quantities of enzymes responsible for bio-degradation of keratin substrates as compared to submerged fermentation process (Belmessikh *et al.*, 2013; Rai *et al.*, 2009; De Azeredo *et al.*, 2006).

The investigation showed that the maximum degradation of chicken feathers was at 40 °C and minimum at 20 and 50 °C. Saber *et al.* (2009) showed similar results that keratinolytic activity by *Aspergillus* spp. was highest at 35 °C-40 °C and least at 20 °C and 50 °C temperatures, using chicken feathers as sole source of carbon and nitrogen.

Aspergillus spp. are ubiquitous fungi and are able to grow on natural media without any special requirements. *A. niger* was carefully isolated and was cultured on malt extract and agar medium for the present study. The strain was confirmed by its morphological characteristics under a microscope and was used for bio-degradation of chicken feathers in submerged cultivation process. Friedrich *et al.* (1999) isolated fungi from soil and demonstrated that *A. niger* showed keratinolytic activity using chicken feathers as substrate. Kim (2003) proved that *A. niger* can grow on chicken feather

substrate as sole source of carbon, and has the ability to degrade keratin, they investigated on five *Aspergillus* species their study revealed that *A. flavus, A. niger* and *A. terreus* showed highest keratinolytic activity. Lopes *et al.* (2011) used *A. niger* to degrade chicken feathers in submerged cultivation process and showed that *A. niger* had high keratinolytic activity.

The production of keratinase enzyme by *A. niger* was maximum at 0.5 g/100 mL concentration of substrate (chicken feathers) and minimum at 0.7 g/100 mL of substrate. Similarly, Brandelli and Riffel (2005) estimated that with the increase in substrate concentration there is a slight decrease in keratinase production. The production of keratinase was found to be maximum at pH 7.0. It was estimated that increase in pH caused a slight decrease in keratinase production. Saber *et al.* (2009) also described that highly acidic or alkaline pH caused reduction in the keratinase production by *Aspergillus* spp. and 7-7.5 pH was the most suitable range for maximum keratinase production and biodegradation of the chicken feathers. The production of keratinase enzyme was maximum at 8 g/100 mL concentration of yeast extract and minimum at 4 g/100 mL of substrate. It was found that increase in the yeast extract concentration also increases the keratinase enzyme production. Similar results were found by Mazotto *et al.* (2010), the keratinolytic activity was enhanced by the addition of yeast extract in the medium. However, they used bacterial species and human hair as kertain substrate. Maximum production of keratinase was found in the 50 mL fermentation volume, while minimum production of the enzyme was in 200 mL medium volume. Raju and Divakar (2013) found that by increasing the fermentation medium volume the production of keratinase decreases slightly.

Conclusion

The results suggest that bio-degradation of chicken feathers by *A. niger* can successfully bio-degrade poultry feathers and thus can help to reduce the agro-industrial waste. Moreover, this procedure can prove to be a helpful process for biotechnological processes and for industrial production of keratinase enzyme that can be used in production of several useful commercial products. Furthermore, this process can be a better alternative than conventional methods of management of waste chicken feathers which are energy consuming, less efficient and not environmentally friendly. Therefore, it is recommended that bio-degradation of discarded chicken feathers by micro-organisms should be adopted because chicken feathers are problematic agro-industrial waste of poultry industry. Moreover, we also encourage to conduct research on other fungal as well as bacterial species and identify optimum conditions for efficient and large scale bio-degradation of waste poultry feathers. For a developing country like Pakistan, bio-degradation of chicken feathers by *A. niger* can be promising candidate, because it is a less energy consuming process, cost effective and has numerous industrial and commercial applications.

Conflict of Interest. The authors declare no conflict of interest.

References

Anbu, P., Hilda, A., Sur, H.W., Hur, B.K., Jayanthi, S. 2008. Extracellular keratinase from *Trichophyton* sp. HA-2 isolated from feather dumping soil. *International Biodeterioration and Biodegradation*. **62:** 287-292

Anitha, T.S., Palanivelu, P. 2013. Molecular identification of two new isolates of keratinolytic fungi using internal transcribed spacer regions. *Journal of Applied Sciences in Environmental Sanitation*, **8:** 67-75.

Anson, M.L. 1938. Plant proteolytic enzyme. *Journal of General Physiology*, **22:** 79-84.

Belmessikh, A., Boukhalfa, H., Mechakra-Maza, A., Gheribi-Aoulmi, Z., Amrane, A. 2013. Statistical optimization of culture medium for neutral protease production by *Aspergillus oryzae*. Comparative study between solid and submerged fermentations on tomato pomace. *Journal of the Taiwan Institute of Chemical Engineers*, **44:** 377-385.

Brandelli, A., Riffel, A. 2005. Production of an extracellular keratinase from *Chryseobacterium* sp. growing on raw feathers. *Electron. Journal of Biotechnology*, **8:** 35-42.

Brandelli, A., Sala, L., Kalil, S.J. 2015. Microbial enzymes for bioconversion of poultry waste into added-value products. *Food Research International*, **73:** 3-12.

Bressollier, P., Letourneau, F., Urdaci, M., Verneuil, B. 1999. Purification and characterization of a keratinolytic serine proteinase from *Streptomyces albidoflavus*. *Applied and Environmental Microbiology*, **65:** 2570-2576.

De Azeredo, L.A.I., De Lima, M.B., Coelho, R.R.R., Freire, D.M.G. 2006. Thermophilic protease

production by *Streptomyces* sp. 594 in submerged and solid-state fermentations using feather meal. *Journal of Applied Microbiology*, **100:** 641-647.

Deivasigamani, B., Alagappan, K.M. 2008. Industrial application of keratinase and soluble proteins from feather keratins. *Journal of Environmental Biological*, **29:** 933-936.

Friedrich, J., Gradisar, H., Mandin, D., Chaumont, J.P. 1999. Screening Fungi for synthesis of keratinolytic enzymes. *Lett. Applied Microbiology*, **28:** 127-130.

Kim, J. 2003. Keratinolytic activity of five *Aspergillus* species isolated from poultry forming soil in Korea. *Mycobiology*, **31:** 157-161.

Lopes, F.C., Dedavid e Silva, L.A., Tichota, D.M., Daroit, D.J., Velho, R.V., Pereira, J.Q., Corrêa, A.P.F., Brandelli, A. 2011. Production of proteolytic enzymes by a keratin-degrading *Aspergillus niger*. *Enzyme Research*, 487093.

Matikeviciene, V., Masiliuniene, D., Grigiskis, S. 2009. Degradation of keratin containing wastes by bacteria with keratinolytic activity. *Environment Technology Resources*, **1:** 284-289.

Mazotto, A.M., Cedrola, S.M., Lins, U., Rosado, A.S., Silva, K.T., Chaves, J.Q. Rabinovitch, L., Zingali, R.B., Vermelho, A.B. 2010. Keratinolytic activity of *Bacillus subtilis* AMR using human hair. *Lett. Applied Microbiology*, **50:** 89-96.

Mazotto, A.M., Couri, S., Damaso, M.C., Vermelho, A.B. 2013. Degradation of feather waste by *Aspergillus niger* keratinases: Comparison of submerged and solid-state fermentation. *International Biodeterioration and Biodegradation*, **85:** 189-195.

Rai, S.K., Konwarh, R., Mukherjee, A.K. 2009. Purification, characterization and biotechnological application of an alkaline b-keratinase produced by *Bacillus subtilis* RM-01 in solid-state fermentation using chicken-feather as substrate. *Biochemical Engineering Journal*, **45:** 218-225.

Raju, E.V.N., Divakar, G. 2013. Production of keratinase by using *pseudomonas aeruginosa* isolated from poultry waste. *International Journal of Pharmaceutical, Chemical and Biological Sciences*, **3:** 79-86.

Riffel, A., Brandelli, A., Heeb, P., Lucas, F. 2003. Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. *Arch. Microbial*, **179:** 258-265.

Saber, W.I.A., El-Metwally, M.M., El-Hersh, M.S. 2009. Keratinase production and biodegradation of some keratinous waste by *Alternaria tenuissima* and *Aspergillus nidulans*. *Research Journal of Microbiology*, **5:** 21-35.

Shih, J.C.H. 1993. Recent Development in Poultry Waste Digestion and Feather Utilization. *Poultry Science*, **72:** 1617-1620.

Williams, C.M., Lee, C.G., Garlich, J.D., Shih, J.C.H. 1991. Evolution of bacterial feather fermentation product, feather lyaste, as a feed protein. *Poultry Science*, **70:** 85-94.