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Biosynthesis of keratinase by feather-degrading *Aspergillus niger* under submerged fermentation process

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Abstract : In the present study, keratinase enzyme was produced using *Aspergillus niger* under submerged fermentation condition. Another aim of this study was to degrade the chicken waste found in the local market of Lahore. Various fermentation conditions were optimized such as effect of substrate concentration, various pH levels, incubation temperatures, concentration levels of yeast extract and different volumes of fermentation media. The impact of these factors was investigated to get the maximum keratinase production and highest biodegradation of chicken feathers in fermentation medium. The maximum yield of keratinase production was achieved after adding 6g of chicken feathers, 8g of yeast extract, maintaining pH at 7 and by incubating 150ml of fermentation medium by incubating at 30°C for 5 days. The maximum yield of keratinase enzymes (6.78U/ml) and highest biomass degradation (15.7g/1000ml) was obtained after incubating one litter fermentation medium in one litter of fermentor. The present study identifies the *Aspergillus niger* as a potential candidate for the biodegradation of chicken waste and production of keratinase enzyme under submerged fermentation conditions.
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Introduction

In recent years, poultry industry has expanded a lot due to massive increase in world population, which in turn has increased the problematic waste production from the poultry industry. Discarded chicken feathers from the poultry industry are considered as agro-industrial waste. Waste feathers from poultry industry are a major cause of pollution in the environment. Discarded feather also causes various human diseases like chlorosis, mycoplasmosis and fowl cholera (Williams *et al.*, 1991). The recent outbreak of bird flu caused by H5N1 virus is 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 conventional utilization of chicken feathers include production of animal feed, protein, amino acid source and fertilizers production, etc. which are energy consuming and costly processes and may result into loss of essential amino acids (Deivasigamani and Alagappan, 2008; Papadopoules *et al.*, 1986). Instead of chemical processing, biotechnological processing of feathers for the production of feather meal, is preferred as it preserves the essential amino acids moreover it helps to prevent accumulation of feather in the environment and decrease the development of pathogens (Riffel *et al.*, 2003). 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 keratinolytic enzymes in the fermentation medium supplemented with many ingredients (Anbu *et al.*, 2008). There are various strains of *Aspergillus* sp. which are identified as keratinolytic fungi for the biodegradation of chicken feathers through number of researches and surveys done till present (Saber *et al.*, 2009; Kim, 2003).

The objective of the present study was to investigate the production of keratinase enzyme and biodegradation of the chicken feathers in submerged fermentation process, by a keratinolytic strain of *Aspergillus niger*. A culture medium based on keratinous substrate was selected, and chicken feathers were used as substrate. This study also aimed to

evaluate the effect of the substrate concentration, pH of the fermentation medium, yeast extract concentration, incubation temperature and different volumes of fermentation medium on the production of keratinase enzyme by *Aspergillus niger*. All the data collected through experiments was also statistically analysed.

Materials and Methods

Substrate (Chicken Feathers)

Chicken feathers were collected from the local poultry waste site and used as a substrate for the production of keratinase enzyme through degradation by *Aspergillus niger*. Chicken feathers were cleaned from attached flesh by washing them thoroughly in tap water. The chicken feathers were dried in hot air oven at 50°C for 30 minutes and were cut into tiny fragments and stored at room temperature for further processing.

Procurement of Fungal Strain

Aspergillus niger was taken from the Institute of Industrial Biotechnology, G.C. University Lahore.

Maintenance of Culture

The strain was maintained on MEA slants (2g agar and 2g malt extract was dissolved in 100ml of distilled water for slants preparation). The slant cultures were incubated at 28°C for 5 days to get the maximum growth of *Aspergillus niger*. The fully grown slants were stored in refrigerator at 4°C for further experiments. Sub-culturing was carried out after every 2 weeks and *Aspergillus niger* was frequently examined under a light microscope to avoid any contamination.

Preparation of Spore Suspension

Spore suspension of *Aspergilus niger* was prepared by pouring 10ml of distilled water in a fully grown fungal slant. It was vortex 2 to 3 times for the proper mixing of spores in the distilled water. One millilitre of spore suspension contains $10^{6\text{-}7}$ spores. Spore suspension was stored at 4°C for further use in fermentation medium.

Composition and Preparation of Fermentation Medium

All the chemicals enlisted in Table 1 were dissolved in a 250ml Erlenmeyer flask containing 90 ml of distilled water. The pH of the fermentation medium was maintained at 7 using 1N HCl and ammonia solution. The flask containing the fermentation medium was autoclaved and after sterilization 10ml of spore suspension was added in the fermentation medium.

Table 1: Composition of fermentation medium for the production of Keratinase

Chemicals	g/100ml
Chicken Feathers	0.5
Yeast Extract	2
Peptone	2
Asparagine	2
$CaCl_2$	1
K_2HPO_4	0.2
$ZnSO_4$	0.1
$MgSO_4$	0.1
$FeSO_4$	0.1

The flask was then kept in the shaking incubator rotating at 200 rpm at 28°C for 5 days. Enzyme was extracted by centrifugation of the mixture and cell biomass was weighed after centrifugation.

Standard Curve of Tyrosine

Different concentrations of tyrosine i.e. 20, 40, 60, 80, 100 and 120µg/ml, were prepared in 0.0006N HCl. The 1ml of each dilution was taken in separate test tubes, then 5ml of 0.5M Na_2CO_3 and 3ml Folin-Ciocalteu reagent was added in each test tube. All the test tubes were incubated in water bath at 37°C for 30 min. After incubation, optical density was measured at 550 nm using spectrophotometer. The blank was also prepared by taking 1ml of 0.0006N HCl in a test tube. A linear line was obtained after plotting the data in graph.

Keratinase Assay

Preparation of keratinase extract

Fermentation medium was centrifuged at 4°C for 10 min at 10,000 rpm. Supernatant was collected in a separate glass bottle and the pellet was weighed to find out the final weight of the chicken feather's biomass. The supernatant was stored at 4°C for futher keratinase assay.

Determination of Keratinase Activity

Keratinase activity was determined by following the Anson's method (Anson, 1938). 1 ml of the supernatant was taken in a test tube and 1.0 ml of phosphate buffer (pH of phosphate buffer was maintained at 7) was added in it. 1ml of the 2% tyrosine (pH 7.0) was also added in the buffer-enzyme solution. This was done in duplicate. All the test tubes were incubated at 37°C for 10 minutes in a water bath then 10 ml of 5N TCA (Tri-chloro Acetic acid) was added in the test tubes to stop the reaction. The precipitated keratin was then filtered off. After filtration, 5ml of the filtrate was taken in a test tube and 10 ml of 0.5N Na_2CO_3 solution was added in it. After a few seconds 3.0 ml of the Folin-Ciocalteu reagent was poured in the test tube. It was heated for a

few seconds for color development. After cooling it at room temperature, optical density was measured in spectrophotometer at 550 nm. Blanks of the samples were prepared by using the TCA. One unit of keratinase activity was expressed as 1µml of tyrosine released per minute under the above conditions.

Optimization of fermentation parameters

Various parameters were optimized for the production of keratinase enzyme and biodegradation of chicken feathers using *Aspergillus niger* in submerged fermentation condition.

Effect of various parameters on the biodegradation of the chicken feathers by *Aspergillus niger* using submerged fermentation:

Various concentartions of substrate

Different concentrations of substrate (Chicken Feathers) such as 2, 4, 6, 8 and 10g were used in the Fermentation medium.

Effect of pH

The fermentation media were exposed to different pH values ranging from 2-8 using chicken feathers as substrate.

Various incubation temperatures

The fermentation media were exposed to different incubation temperatures such as 20, 25, 30, 35, 40, 45 and 50°C.

Various concentrations of yeast extract

Different concentrations of yeast extract were added in the reaction mixture ranging from 2-8g.

Different volumes of fermentation media

Various volumes of fermentation media were prepared ranging from 50ml to 300ml. The pH of all the fermentation media was maintained at 7.

Statistical Analysis

The data collected was analysed statisticaly using Analysis of Varience (ANOVA) with the help of statistical software, "IBM SPSS Statistics ver. 20".

Results and Discussion

Effect of different Concentrations of Substrate

For evaluating the effect of different concentrations of chicken feathers such as 2, 4, 6, 8 and 10g were used in fermentation medium as substrate for the biodegradation and production of keratinase by *Aspergillus niger*. From the 6g of chicken feathers present in fermentation medium, the highest keratinase activity was obtained (0.68±0.05 U/ml) at 0.5g while lowest production of enzyme (0.35±0.03 U/ml) was obtained from 10g of chicken feathers as presented in Table 2. Highest degradation of chicken feathers was also observed in 6g substrate of chicken feathers (0.91±0.03g/100ml).

Table 2: Effect of different concentrations of substrates on the production of keratinase and biodegradation of chicken feathers

Chicken Feathers (g)	*Aspergillus niger*	
	Keratinase production (U/ml)	Cell Biomass (Degradation of chicken feathers (g/100ml)
2	0.35±0.01	1.5±0.01
4	0.45±0.02	2.5±0.02
6	**0.68±0.01***	**0.91±0.03***
8	1.35±0.02	0.96±0.01
10	1.00±0.05	3.5±0.02

Each value is an average of three replicates and ± indicates the standard deviation of these replicates.

Effect of pH

To determine the effect of different pH values on the production of keratinase enzyme and biodegradation of chicken feathers by *Aspergillus niger*, the various fermentation media were prepared having different pH values such as 2, 3, 4, 5, 6, 7 and 8. Maximum keratinase production was achieved at pH 7 (1.9±0.01 U/ml) of the fermentation medium and lowest keratinase was achieved at pH 5 (0.16±0.2 U/ml). Maximum biodegradation of chicken feathers was found in the fermentation medium having pH 7 (0.52±0.01g/100ml) as described in the Table 3.

Table 3: Effect of pH on Keratinase production and biodegradation of chicken feathers

pH	*Aspergillus niger*	
	Keratinase production (U/ml)	**Cell Biomass (Degradation of chicken feathers (g/100ml)**
2	0.39±0.01	1.2±0.01
3	0.32±0.02	1.4±0.02
4	0.41±0.01	0.81±0.03
5	0.16±0.02	0.97±0.01
6	1.1±0.05	0.88±0.02
7	**1.9±0.01***	**0.52±0.01***
8	1.5±0.02	1.1±0.01

Each value is an average of three replicates and ± indicates the standard deviation of these replicates.

Effect of Incubation Temerpatures

Table 4 indicates that fermentation media were incubated at various temperatures to obtain the enhanced yield of keratinase and maximum biodegradation of chicken feathers using *Aspergillus niger*. The highest production of enzyme was found after incubating the fermentation medium at 30°C (0.91±0.01 U/ml) while the lowest yield was obtained from the fermentation medium incubated at 50°C (0.05±0.02 U/ml). Chicken feathers were degraded at their maximum at 30°C (0.75±0.03g/100ml).

Table 4: Effect of incubation temperatures on keratinase production and biodegradation of chicken feathers

Incubation Time (°C)	*Aspergillus niger*	
	Keratinase production (U/ml)	**Cell Biomass (Degradation of chicken feathers (g/100ml)**
20	0.19±0.01	0.99±0.03
25	0.82±0.02	0.98±0.02
30	**0.91±0.01***	**0.75±0.03***
35	0.52±0.02	0.97±0.01
40	0.32±0.05	1.80±0.01
45	0.19±0.01	1.52±0.01
50	0.05±0.02	1.2±0.05

Each value is an average of three replicates and ± indicates the standard deviation of these replicates.

Effect of various concentrations of yeast extract

Table 5 representing the impact of various concentration levels of yeast extract on the yield of keratinase and degradation of chicken feathers. Yeast extract is a nitrogen source, it enhances the growth of fungus in the fermentation medium. The highest keratinase production was achieved when 7g (2.6±0.01 U/ml). The least enzyme production was found from the fermentation medium incubated with 3g (0.61±0.01 U/ml) of yeast extract. The maximum biodegradation was achieved from fermentation medium incubated with 8g (0.41±0.01g/100ml**)** of yeast extract.

Table 5: Effect of various concentrations of yeast extract on keratinase production and biodegradation of chicken feathers

Yeast Extract (g)	*Aspergillus niger*	
	Keratinase production (U/ml)	Cell Biomass (Degradation of chicken feathers (g/100ml)
2	0.72±0.01	0.93±0.01
3	0.61±0.01	0.78±0.01
4	0.99±0.03	1.12±0.02
5	1.32±0.02	1.25±0.01
6	1.56±0.01	1.92±0.01
7	**2.6±0.01***	0.97±0.01
8	2.13±0.02	**0.41±0.01***

Each value is an average of three replicates and ± indicates the standard deviation of these replicates

Effect of different volumes of fermentation medium

Effect of different volumes of fermentation medium were employed to get the enhanced yield of keratinase enzyme and to determine the maximum degradation using *Aspergillus niger*. The enhanced yield of keratinase enzyme was obtained from the 150ml (2.31±0.03U/ml) of fermentation medium and lowest yield was achieved from 50ml (1.32±0.01U/ml) of fermentation medium. Highest degradation of chicken feathers was observed in 300ml of fermentation medium (0.56±0.01U/ml) as described in Table 6.

Table 6: Effect of different volumes of fermentation medium on keratinase production and biodegradation of chicken feathers

Volumes of fermentation medium (ml)	*Aspergillus niger*	
	Keratinase production (U/ml)	Cell Biomass (Degradation of chicken feathers (g/100ml)
50	1.32±0.01	1.33±0.01
100	1.56±0.01	1.39±0.01
150	**2.31±0.03***	0.94±0.02
200	2.12±0.02	0.89±0.01
250	1.90±0.01	1.12±0.01
300	1.92±0.01	**0.56±0.01***

Each value is an average of three replicates and ± indicates the standard deviation of these replicates

Biodegradation of chicken feathers in fermentor

After optimizing various fermentation parameters, optimum values were used in fermentation medium to get the maximum degradation in one litter fermentor. The pH of the fermentation medium was maintained at 7 using 1N HCl and ammonia solution and it was incubated at 25°C for 5 days. After 5 days, maximum biodegradation of chicken feathers was achieved (15.7g±0.01 g/litter) and an enhanced production of keratinase enzyme was achieved (6.78±0.01 U/ml).

Discussion

Feather degrading microorganisms are of great significance in reducing the environmental pollution caused by open dumping of chicken feathers. It is also of great importance in improving the live stock feed and production of keratinase enzyme of industrial importance. Annually, tons of feather waste is being discharged in the environment and on these sites microorganisms help to minimize the pollution by producing pure keratinase enzyme. The keratinase enzyme is a part of cosmetics and pharmaceutical industry these days. The present study was designed to identify the potential candidate for the maximum biodegradation of feather waste in laboratory under submerged fermentation conditions.

The investigation showed that the maximum degradation of chicken feathers was achieved at 30°C using pH 7 in the fermentation medium. Saber *et al.* (2009) worked on keratinolytic activity using the fungal strain of *Aspergillus* sp. and found maximum yield of keratinase enzyme and biodegradation of

chicken waste at 35°C and least yield and biodegradation was observed at 20°C and 50°C temperatures.

The production of keratinase enzyme by *Aspergillus niger* was maximum at 6g/100ml concentration of substrate (chicken feathers) and minimum at 2g/100ml of substrate (Table 2). Brandelli and Riffel, (2005) also described that with the increase in substrate concentration there is a slight decrease in keratinase production in the fermentation medium.

In the present study, the production of keratinase was found to be maximum at pH 7.0 (Table 3). 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* sp. 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 7g/100ml concentration of yeast extract and minimum at 3g/100ml of substrate (Table 5). 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.

Maximum production of keratinase was found in the 150ml fermentation volume while minimum production of the enzyme was in 50ml medium volume. Raju and Divakar (2013) found that by increasing the fermentation medium volume the production of keratinase decreases slightly.

Conclusion:

Aspergillus niger was able to biodegrade chicken feathers and produced keratinase enzyme. *Aspergillus niger* can prove to be an ideal fungal strain for the reduction of waste chicken feathers from poultry industry, moreover, this process can be useful for the production of keratinase enzyme and other industrial products such as, amino acids, glues, animal feed, etc.

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