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Isolation, Identification and Production of Keratinolytic Proteases by a Chicken Feathers – Degrading *Aspergillus niger* Strain

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A B S T R A C T

Proteolytic fungi was isolated from soil sample collected from nearby area of Acentric Biotech and Research Laboratory, Phase 7, Industrial area, Mohali, (Punjab)on potato dextrose agar media plate containing chicken feathers as cheap and local, protein substrate. The chicken feathers sample was collected from village butchery, phase 5, Mohali (Punjab).The fungi was inoculated in basal for the production of protease enzyme. The crude enzyme extract identified by Bradford protein assay method. The protease enzyme activity of *Aspergillus niger* was 58.33 µ/dL for test sample and 29.37 µ/dL for control. The maximum extracellular protease production was observed at 25 °C.

Introduction

Aspergillus niger

Aspergillus niger, a species of the genus *Aspergillus*, is a filamentous fungus that grows aerobically on organic matter such as litter, compost, decaying plants, and animal material present in the soil. *Aspergillus niger* can grow in a wide range of temperatures of about 6-47°C with an optimum temperature range of about 35-37°C. The pH required for its growth is about 1.4 - 9.8 and water activity for growth is 0.88. This fungus is non-pathogenic and regarded as a safe organism as reported by FDA (Food and drug administration) because it is widely distributed in nature and humans are exposed to it every day without any disease becoming apparent.

Aspergillus niger can produce citric acid and enzymes such as protease, oxidase, alpha-amylase, and glucoamylase that has various industrial applications. *Aspergillus niger* strain has the potential to utilize the chicken feathers' keratin. (Schuster *et al.*, 2002)

Protease Enzyme

Proteases are the enzymes that catalyze the hydrolysis of peptide bonds present in proteins and

polypeptides and are classified based on their origin, catalytic activity, and the nature of reactive groups in their catalytic sites. These are divided into two groups endo-peptidases and exo-peptidases. The major source of protease enzymes is animals, plants, and microorganisms like bacteria (example: *Bacillus subtilis* & *Bacillus licheniformis*) and fungus (example: *Aspergillus niger*).

Proteases have various applications such as in baking industries for the production of bread, waffles, baked food, and crackers that reduces the mixing time, decreases dough consistency and uniformity as well as regulates the gluten strength in bread, and improve texture and flavor. In the food industry, it is in use to improve the flavor, nutritional value, solubility, and digestibility of food proteins and to modify the functional properties that include coagulation and emulsification. (Raveendran Sindhu *et al.*, 2018)

Chicken feathers

The feather is a complex ectodermal organ with a hierarchical pattern. These are excellent models for morphogenesis studies because of their accessibility, and their distinct patterns can be used to assay the role of specific molecular pathways. They play the main role in endoderm, communication, and flight.

Poultry feathers have two important parts consisting of feather fibers and quill out of which feather fibers are made of proteins known as hydrophobic keratin that varies with ecological poultry farm facilities. Poultry farms are about 135 mm long with a density of around 0.8 g/cm^3 and are known to be lighter material. The feather's fiberis about 5 μm in diameter with a length of about 3-13 mm. In poultry industries, chicken feathers are the major waste and 90 % of pure keratin can be obtained from feather waste. (Randall B. Widelitz *et al.*, 2007)

Submerged fermentation method

The common sense of fermentation is the conversion of sugar into an organic acid or alcohol that occurs naturally in many foods and humans. Fermentation involves the intentional use of microorganisms such as bacteria like, yeast, and fungi to make products such as enzyme metabolites and recombinant products that are useful to humans on an industrial scale. The main center for fermentation are bioreactors and are based on various parameters such as the type of the substrates used, environmental parameters, and the organisms being used for the fermentation process. (Tomas Branyik *et al.*, 2013)

It is divided into 2 types namely submerged fermentation (SMF) and solid-state fermentation (SSF). Solid-state substrates use solid substrates such as bran, bagasse, and paper pulp that are utilized very slowly and steadily, so the same substrate can be used for long fermentation periods while free-flowing liquid substrates such as molasses and broths are used are utilized quite rapidly hence need to be supplemented with nutrients.

The bioactive compounds such as enzymes are secreted into the fermentation broth. This type of fermentation technique is best suited for microorganisms such as bacteria that require high moisture content such as fungi. (Subramaniyam *et al.*, 2012)

In the present research, the strain *Aspergillus niger* strain was used as a degrading fungus to carryout keratinolytic activity using chicken feathers as substrate to produce protease enzyme to use it as an natural food additive such as in fruit jellies and jams.

Materials and Methods

Sample collection for Fungus

Sample for fungus *Aspergillus Niger* was collected from the nearby area of the building of Acentric Biotech and Research Laboratory, D – 108 E, First Floor, Phase 7, Industrial Area, Sahibzada Ajit Singh Nagar, Punjab 160055. The sample was

collected in a clean sterile plastic beaker with the help of a clean spatula and was brought to the laboratory for microbial analysis.

Isolation of fungus from soil sample

25 ml of Potato dextrose agar (PDA) media was prepared by using 0.1 g of potato starch, 0.5 g of dextrose, and 0.38 g of agar with pH maintained at 5.6 ± 0.2. The ingredients were mixed in distilled water and then autoclaved at 121°C at 15 psi pressure for 15 minutes. After autoclaved media was kept in laminar airflow swirled to ensure proper mixing and poured into a petri plate for solidification. The soil sample collected was serially diluted and 10^{-5} dilution was used for spreading over the PDA plate. After spreading the sample, plates were kept at 25°C in incubator for 5–7 days.

Identification of *Aspergillus niger*

For identification, glass slides were taken and a drop of distilled water was poured on the glass slide and a hyphae with the help of sterilized inoculums loop was placed on water droplet and allowed to dry to make a smear. 2-3 drops of Lactophenol cotton blue (LPCB) stain were poured on the smear and the slide was examined with the help of a compound microscope under a 40 X lens to observe the morphological characteristics, such as hyphae, spores, and conidial heads,

Preparation of broth

Potato dextrose broth was prepared by using 0.04 g of potato starch, and 0.2 g of dextrose with pH maintained at 5.6 ± 0.2. The ingredients were mixed in distilled water and then autoclaved at 121°C at 15 psi pressure for 15 minutes. After autoclaving media, the broth was kept to cool down while exposing the media under UV light and swirled to ensure proper mixing. And a specimen of *Aspergillus niger* with the help of a sterilized inoculating loop was inoculated into the broth and kept for incubation for 5-7 days at 25°C in an incubator to obtain a pure culture of fungus *Aspergillus niger.*

Chicken Feather's sample collection

Feathers sample was collected from Village Butchery, Shop No 132, 133, Phase 5, Sector 59, Sahibzada Ajit Singh Nagar, Punjab 160059 (INDIA). Feathers are the main source of keratin and act as substrate for the keratinolytic activity by the *Aspergillus niger* strain. These feathers were soaked in water for 25minutes and then washed with a 0.1 % "tween 20" solution to wash the feathers from bloodstains, oil and grease, and dust. These washed feathers were spread over newspaper and air dried. These were then chopped into small pieces dipped into distilled water in an Erlenmeyer flask and were autoclaved at 121°C at 15 psi pressure for 15 minutes. After autoclaving these feathers were then kept into the laminar airflow and with the help of sterilized forceps, the feathers were kept gently on blotting paper allowing them to dry.

Submerged fermentation method

For submerged fermentation, 200 ml of basal media was prepared by dissolving 0.02 g of potassium di-hydrogen phosphate ($KHPO_4$), 0.002 g calcium chloride, 0.02 g of ferric sulfate, 0.001 g of zinc sulfate in distilled water with Ph maintained at 7.5 and 100 ml of basal media was transferred to two Erlenmeyer flasks and then autoclavingat 121°C at 15 psi pressure for 15 minutes. After autoclaving, it was kept in laminar airflow to cool down, while exposing it to UV light. 10 grams of feathers were weighed and transferred to each of the 100 ml basal media Erlenmeyer flasks. With the help of the inoculaing loop, one flask was inoculated with *Aspergillus niger* strain while the other flask without inoculated strain was kept as control. These flasks were then kept for 5-7 days for fermentation along with continuous stirring on a rotary shaker at 120 rpm at 25°C.

Microscopic observation of chicken feathers

To check the degradation of chicken feathers submerged in both control and test sample both samples were filtered with the help of filter papers,

and the liquid portion was collected in glass vials while filtered chicken feathers were transferred to blotting paper for drying. Three glass slides were taken with a drop of distilled water laid on each slide. Chicken feathers each from standard, control, and test sample were placed on glass slide. These chicken feathers were then observed with a compound microscope under a 40 X lens.

Bradford protein assay test for detection of protease enzyme

To test whether the protease enzyme is present in the test sample and control, a highly sensitive Bradford protein assay test was performed. A pinch of bovine serum albumin (BSA) was used as a standard protein. 1 ml of control, test sample solution, and standard in three separate test tubes were mixed with 1 ml of Bradford reagent. On mixing, change in color of the solution shows the presence of protein in the solution.

Protease assay method

To check the production of protease enzyme in the test sample and control solution5 ml of (0.65 %) case in solution was taken in two test tubes and kept in a water bath at 37° C for 5 minutes. Meanwhile, enzyme solution was prepared by mixing 2 ml of calcium sodium buffer (10 mM Sodium acetate + 5 mM calcium carbonate) and then 1 ml control and sample solution was taken in those two separate eppendorfs well labeled and mixed thoroughly by shaking and then incubating them for 10 minutes at 37° C. After incubation 5 ml of (110 mM) tri-chloro-acetic acid was added to stop the reaction. Again was incubated at 37°Cfor 30 Minutes.

After incubating the solutions in two different eppendorfs were filtered with the help of filter paper. 2 ml of filtrate from two different eppendorfs i.e of control and sample was taken in two test tubes and then 2.5 ml of (0.5) Sodium carbonate was added to each of them. Also, 1 ml of freshly prepared (0.5 M) FC reagent was added. These solutions were then mixed by shaking and were incubated at 37°C for 30 minutes. After incubation of both test control and test samples, absorbance at 660 nm with the help of spectrophotometer was recorded.

Results and Discussion

The strain of fungus *Aspergillus niger* is a filamentous fungus that is white to yellow in color during its initial growth and changes to black after a few days of growth along with the formation of conidial spores. It was isolated from a soil sample that was serially diluted and 10^{-5} dilution was used for spreading over the PDA plate. The colony morphology study showed it has a cottony appearance and the edges of the colony on Potato dextrose agar (PDA) medium appears to be pale yellowish producing radial fissures. The colonies were of different sizes, some small, some medium, and some large colonies were found. After staining with Lacto-phenol cotton blue and microscopy, it was observed that all *Aspergillus niger* isolates have smooth colored conidiophores and conidia when observed with the compound microscope at 40X.

200 ml of Basal media also known as general-purpose media was prepared. 100 ml of basal media was kept as control while another 100 ml of basal media with inoculated *Aspergillus niger* strain and submerged chicken feathers as substrate in both Erlenmeyer flasks were kept for fermentation for 5-7 days with continuous shaking on a rotary shaker at 25°C.

With time during the fermentation process, the basal media of the test sample with inoculated microorganisms started turning turbid, and also visible hyphae and mycelium were seen in test sample Erlenmeyer's flask. During this time the fungi exhibits its capabilities to degrade chicken feathers by performing keratinolytic activity and secreting enzymes such as proteases. After the fermentation period was over both control and test samples were filtered with the help of filter paper. Feathers were allowed to dry while liquid portion was collected in a glass vial.

Table.1 Readings on spectrophotometer at 660 nm

S. No	Samples	Readings
1.	BLANK	**1.423**
2.	CONTROL	**2.015**
3.	SAMPLE	**1.340**

Table.2 Enzyme activity in μ / dL

S. No	Samples	μ / dL
1.	CONTROL	**58.33**
2.	TEST SAMPLE	**29.37**

Fig.1 (a), (b).Areas from where soil sample was collected

Sample collection for fungus

Fig.2 Soil sample collected in the sterile plastic beaker

Sample collection for fungus

Fig.3 Serially diluted soil sample

Growth of fungus

Fig.4 (a),(b). Growth of fungus at 25°C on PDA plate

Growth of fungus

Fig.5 (a), (b). Microscopic view of *Aspergillus niger* under 40X lens

Identification of *Aspergillus niger*

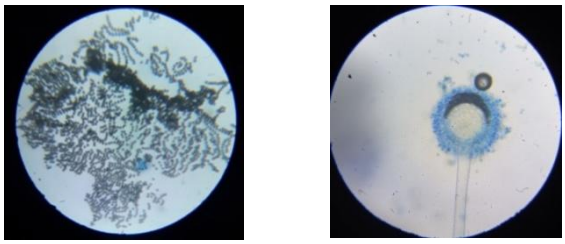


Fig.6 Growth of fungus *Aspergillus niger*

Broth preparation

Fig.7 (a), (b), (c). Chicken Feathers collected from meat processing shop

Feathers sample collection

Fig.8 (a), (b). Autoclaved chicken feathers

Feathers sample collection

Fig.9 Autoclaved control and sample basal media

Submerged fermentation

Fig.10 Chicken feathers submerged in control and test sample

Submerged fermentation

Fig.11 Control and test sample on rotary shaker at 120 rpm

Fig.12 Control and test sample after fermentation

Fig.13 (a), (b), (c). Filtered feathers and liquid portion of control and test sample

Fig.14 Glass slides prepared for chicken feathers examination

Examination of the degradation of chicken feathers by *Aspergillus niger* strain using a compound microscope

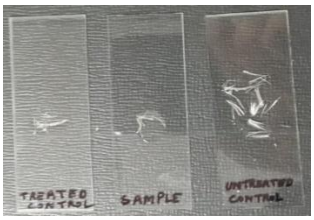


Fig.15 Standard untreated chicken Feathers

Examination of the degradation of chicken feathers by *Aspergillus niger* strain using a compound microscope

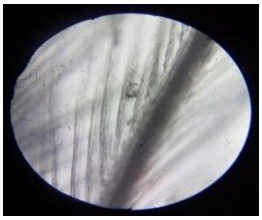


Fig.16 Chicken feathers in the control sample

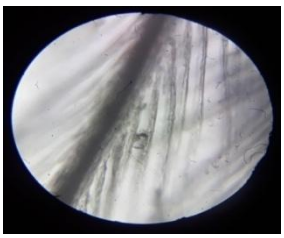


Fig.17 Chicken feathers in the test sample

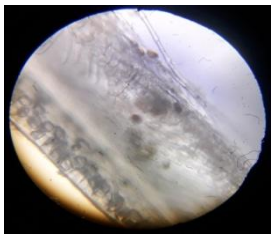
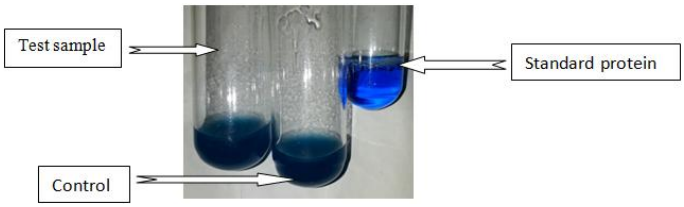


Fig.18 Bradford protein assay test

Colorimetric test for detection of protease



Single hair of chicken feathers from standard, control and test samples were visualized with compound microscope with a 40 X lens it was found that chicken feathers in test sample were degraded by fungus as the structure of the feathers was distorted when compared with standard and control chicken feathers as shown in figure 13, 14, 15.

To check the presence of proteins in control and test sample solutions a qualitative Bradford reagent test was performed. Bovine serum albumin (BSA) was considered as standard protein for comparison. When control and test samples were treated with Bradford reagent color of sample changes to blue that shows the presence of protein in samples. The Blue color of test sample was fade when compared with compared with standard and darker as in comparison with control sample. This test verified that there is a presence of protein (enzyme) in test sample.

After confirming the presence of protein in control and test sample, next step performed was to check the amount of protease production in test sample and control therefore protease assay test was performed in which reading were obtained with help of spectrophotometer at 660 nm. The reading were obtained on spectrophotometer as shown in table no.1.The amount of enzyme production in µ / dL was calculated by using the formula:

$$\text{Enzyme activity (µ / dL)} = \frac{Absorbance\,of\,Blank - Absorbance\,of\,sample \times 1000}{Absorbance\,of\,Blank}$$

It was seen that production was more in case of test sample as in comparison with control as shown in table no 2.

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