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Studies on Keratinase Producing Fungi Isolated From Poultry And Barber's Dump and their Enzymatic Activity

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ABSTRACT

Keratins are the most abundant proteins in epithelial cells of vertebrates. The keratinise wastes are produced in large quantities all over the world from animals and birds including human beings. Worldwide poultry processing plants are producing millions of tons of feathers as waste product annually, which consists of approximately 90% keratin. The present study investigated keratinises from the decaying poultry feather and human hair samples. Two types of indigenous fungi were isolated and their enzyme activity was measured. The fungi were named as *Aspergillus sp* and *pencillium sp*. These fungi were then tested for the production of amylase, protease, lipase and keratinase**.** Both the fungi were to produce amylase, protease and keratinase. The extracellular lipase was more found to be *Aspergillus sp* when compared to *pencillium sp*. Substrate decomposition was found to be better in *Aspergillus sp* than in *pencillium sp*. *Aspergillus sp* showed 38% loss of hen feather as compared to 25% loss of human hair whereas *pencillium sp* showed 21% loss of feather and 20% of human hair. Two fungi were tested for the production of amino acid by thin layer chromatography. Comparatively, *Aspergillus sp* was found to be producing more amounts of enzymes.

Keywords: Keratin, *Aspergillus sp,* Hair, feather, *pencillium sp*

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INTRODUCTION

Feather and hair are the sources of keratin. They are thrown into the environment as wastes and non-usable commodity. Release of these feathers and hairs in large amount creates pollution problem. High protein content of keratin waste can be used as a good source of protein and amino acids by systemic recycling. Recycling of feather and hairs can provide a cheap and alternative protein feed stuff. Natural degradation of poultry feathers was observed in many studies[1]. A major component of feather and hair is keratin. Because of high degree of cross linking by cysteine disulphide bonds, hydrogen bonding and hydrophobic interactions, it is insoluble. It is not degradable by proteolytic enzyme such as trypsin, pepsin and papain[2]. Keratin structure-chicken feathers are composed of 90% keratin protein, 8% water and 1 % lipid. It is also the main protein of hair, nail hoofs and feathers. Animal hair and wool contain β-keratin whereas birds' feathers contain α-keratin. The polypeptides in α-keratin are closely associated with pairs of β helices, whereas α-keratin has high proportion of β-pleated sheets. Keratins are insoluble macromolecules. They provide a tight packing of polypeptide chains consisting of cystein disulphide bonds .Hence a keratinous material is a tough and protease resistant.

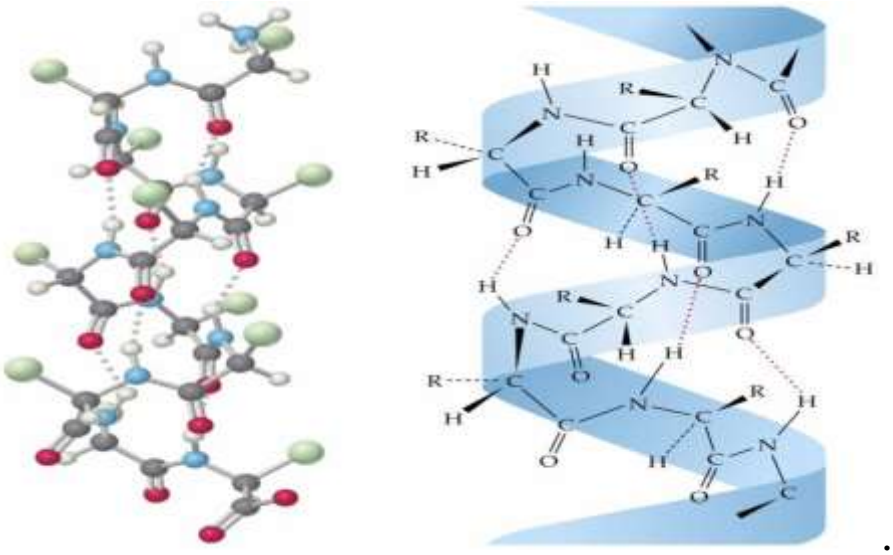

Figure 1 Keratin structure

Keratinise enzyme is a proteolytic enzyme capable of hydrolyzing keratin[3]. This enzyme selectively degrades beta keratin found in feathers. β- keratinase is an enzyme capable of degrading β-keratin[4]. Microorganisms capable of producing keratinize, degraded keratin and converting it into rare amino acids, such as serine, cysteine and proline. Due to strength and stability of keratin, very few micro-organisms are able to break it down and utilize it. There are certain fungi which degrade the protein keratin present in the hair, those fungi are called keratinophilic fungi. There are three important species of kertinophilic fungi namely

chrysosporiumanamorpha, *chrysosporiumkeratinophylum* and *chrysosporiumpannourm.* Kertinolytic fungi take more or less 40 days to degrade feathers. So it was necessary to hasten the degradation process. Hence attempts were made to hasten this with the help of keratinophilic bacteria. The objective of the present study was to isolate and identify keratinophilic fungi from soil. A detailed study was done on the rate of substrate decomposition.

MATERIALS AND METHOD

The poultry waste and barber's dump soil were collected from various area of Tirupathi Urban A.P. Samples were collected from 3 to 4 cm depth contained decaying feathers and hairs were transferred into sterile plastic bags for further study.

Isolation of Keratinolytic Fungus

Hair bating technique was employed to isolate kertinophillic fungi[5]. Isolation of fungi was performed by plating methods on Rose Bengal agar medium. Decaying feather soil samples were taken in half filled sterile petri dishes. Sterilized defatted human hair was placed over the surface of the soil. 10-15 ml of sterile water was added to the soil to facilitate germination of fungal spores. Antibiotic amoxicillin was added to prevent bacterial growth. The preparations were incubated at room temperature at 20-25˚C in the dark for 4-6 weeks. The plates were periodically examined for the development of mycelium. The hairs with fungus growth were removed and placed in the plate of rose Bengal agar medium; kertinolytic activities of fungus were defatted as clear zone around the colony after incubation for up to 5 weeks at room temperature. The selected kertinolytic fungi spores were suspended in the distilled water and stored for further use.

Identification of Isolated Kertinolytic fungus

Staining with lactophenol cotton blue and microscopic observations were performed to identify the isolated fungal culture.

Production and extraction of enzymes from isolated Kertinolytic fungus

After incubation, to evaluate the kertinolytic potential, the selected fungi were grown in flask containing basal medium along with human hair or feathers as a keratin source. 50 ml of basal salt solution was prepared and sterilized at 121˚C for 15 minutes. pH was adjusted to 7.6. The fungal culture were inoculated and incubated at 25˚C for10 days. Culture was filtered aseptically and centrifuged at 4000 rpm for 5 minutes. Supernatant liquid was used as the enzyme extract.

Estimation of Kertinolytic activity

Kertinase activity was determined by using standard method[6]. The culture medium was filtered through whatmann no:1 filter paper to remove residual non degraded feathers and

mycelium. The total weight of substrate was determined and compared with weight of substrate in the control.

Enzyme assay of Kertinase[7]

Kertinase activity was assayed by using human hair. The reaction mixture consisted of 2.6 ml of 0.056 M phosphate buffer (pH 7.6), one gram of hair powder and 0.4 ml of enzyme extract. It was incubated at 34˚C for 1 hour. Reaction was terminated by the addition of 0.612 M trichloroacetic acid and the mixture was chilled immediately to 0˚C and the solution was kept at the same temperature for 30 minutes and filtered. The absorbance of the clear filtrate was measured at 280 nm against reaction blank in which trichloroacetic acid was added before the enzyme source.

Estimation of Amylase enzyme production (DNSA method) [8]

Maltose standard solution was prepared in the concentration of 1mg/ml. From, that 0.1, 0.2 …1 ml of maltose solution was taken in a series of test tubes. Then the solution was made up to 1 ml with distilled water. The mixture was incubated for 10 minutes. After incubation 2 ml of dinitro salicylic acid was added. The blank (1 ml of distilled water + 2ml of DNSA) was also maintained. The mixture was kept in a boiling water bath for 10 minutes. After cooling the test tubes were made up to 10 ml with distilled water. The OD values were measured at 530 nm and the values were recorded. The standard graph was plotted.

Enzyme assay of Amylase

Culture filtrate was obtained by centrifuging the culture at 5000 rpm for 15 minutes. 0.5 ml of culture filtrate was taken and then 1ml of starch solution was added and incubated for 10 minutes at 37 ˚C. 2 ml of DNSA solution was added then the mixture was mixed well and treated in a boiling water bath for 5 minutes. After heating the mixture was cooled then 1ml of distilled water was added. Upon each addition the mixture was mixed well. The OD value was read at 650 nm and the readings were recorded. The standard graph was plotted and the protein content was estimated.

Estimation of Protease enzyme production[9]

0.1,0.2……1 ml of BSA standard solution (1mg/ml) was transferred into a series of 10 ml standard flask and 1 ml of distilled water was added.. To this, 5 ml of alkaline copper reagent was added and incubated for 15 minutes, 0.5 ml of folins phenol reagent was added and incubated at room temperature for 30 minutes. The OD values were measured at 650 nm and the values were recorded. The values were used to plot a standard graph.

Estimation of Protease content

Culture filtrate was obtained by centrifuging the culture at 5000 rpm for 15 minutes. 0.5 ml of culture filtrate, 1 ml of skimmed milk solution and 0.5 ml of mellcaines citrate buffer was

added and incubated for 20 minutes. After incubation 4 ml of 5% trichloroacetic acid was added and the mixture was then filtered. 1 ml of the filtrate, then 5ml of alkaline reagent was added and incubated for 30 minutes. The OD value was read at 650 nm and the reading were recorded. The values were used to plot a standard graph.

Estimation of Substrate decomposition and change in pH

100 ml of basal salt medium was transferred into a series of ten 250 ml Erlenmyer flask then it was sterilized. One gram of hair was added along with an agar disc of (5mm) test fungi and incubated in rotary shaker. pH of the medium and weight loss of hair was estimated at an interval of 0^{th}, 7^{th}, 14^{th} , 21^{th} and 28^{th} days and the results were tabulated. Simultaneously, the same procedure was used instead of 1gm of hair; 1gm of feather was used.

Analysis of Amino acids by thin layer chromatography

Slurry of silica gel was prepared with water. 0.2 mm thick layer of the slurry was applied to the glass plate with TLC applicator. The thickness could be increased up to 2 mm depending upon the substrate used. The coated plates were dried and activated at 0˚C in hot air oven for 24 hours. 2- 2.5 cm from the bottom of the plate using a capillary tube the crude enzyme extract was spotted on TLC plate. The separation was carried out in a glass tank that contains the solvent (mobile phase Butanol: acetic acid: water 32:8:8 % v/ v/ v) about 1.5 - 2cm depth. Mobile phase was allowed into glass tank for 2 hours with a lid over the tank to ensure that the atmosphere within the tank becomes saturated with the solvent mixture. The lid was removed after saturation and the silica gel coated plate containing the sample was developed. The chromatogram was developed until the solvent front was 3-5cm from the top edge. The chromatogram was removed from the chamber and the solvent front was marked with a pencil and dried with air. The chromatogram was sprayed with ninhydrin reagent to detect the primary and secondary amines. After spraying, the chromatogram was placed in hot air oven at 110˚C for 5 minutes. The colors developed at various spots were circled with a pencil. The R_f value was calculated. The amino acid present in the sample was identified. The sample containing amino acid was matching the R_f value with standard amino acid.

RESULTS AND DISCUSSION

Two types of keratinolytic fungi were isolated as pure cultures by hair bating technique. Based on staining and microscopic observations of the cultures, they were identified as *Aspergillus sp* and *pencillium sp* (figure 2 & 3).

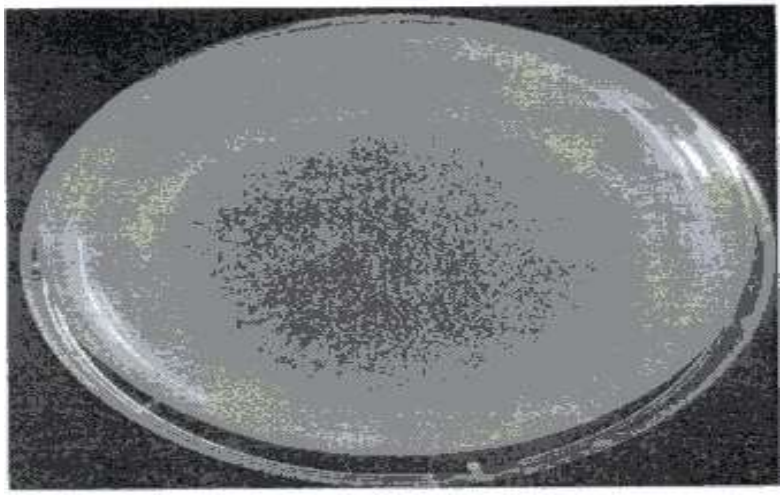

Figure 2 Petri plate containing Organism of *Aspergillus sp*

Figure 3 Petri plate containing Organism of *pencillium sp*

Pugh 1970[10] reported other keratinophilic fungi such as *Microsporum*, and *Cladosporium* degrades more keratin substrates. Production of keratinase was taken at the end of incubation period for the determination of keratinase activity and enzyme assay. Keratinase activity was determined by using the standard method. If the substrate with mycelium weighed more than the weight of the control, fungi were considered to be non keratinolytic, because the increased weight was attributed to the culture filtrate production due to utilization of nutrients present in the basal salt solution and the substrate. If the substrate with mycelium weighed less than the control fungi was considered to be keratinolytic. The net loss of the substrate caused due to the activity of keratinase, produced by the fungi was attributed to release of soluble proteins and peptides from keratinase substrate. This study reveals the substrate mycelium weighed less than the control. One unit of enzyme activity was defined as the amount of enzyme that could liberate products having an absorbance of 0.1. The specific

activity was the number of units per mg of protein. The culture filtrate containing protein was measured by colorimetrically (figure 3).

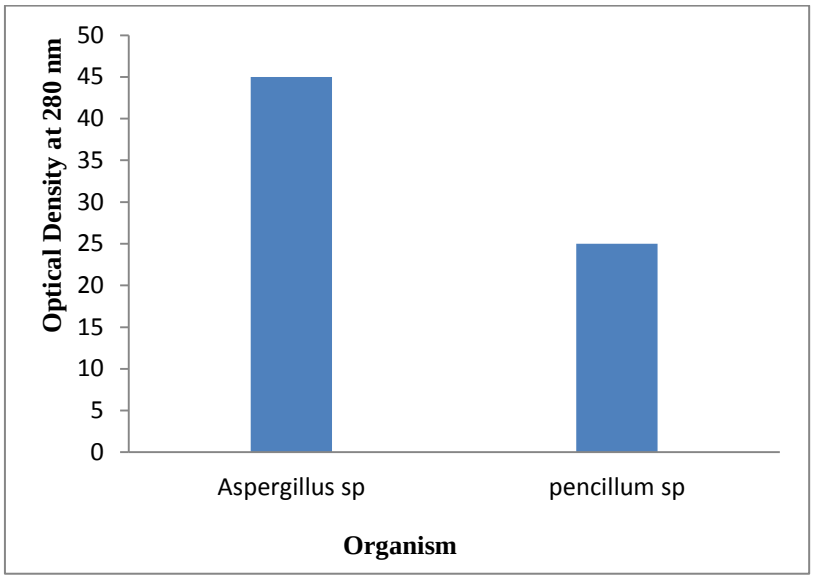

Figure 4: Keratinase Activity

The concentration of amylase and protease enzymes was determined by comparing the standard graphs (figure 4&5). The fungi caused loss in weight of hair and feathers in 28 days of incubation at 28 ˚C. The two isolates degraded large amounts of hen feather than human hair. *Aspergillus sp* showed 38% loss of hen feather as compared to 25% loss of human hair whereas *pencillium sp* showed 21% loss of feather and 20% of human hair. Shih et al reported (1993)[11] 13.5% weight loss in human hair by using *Chrysosporium sp*. Free amino acids were detected in the culture filtrate by thin layer chromatography. The free amino acids were determined by calculating their R_f values. Culture filtrate of *Aspergillus sp* was loaded, three amino acids were

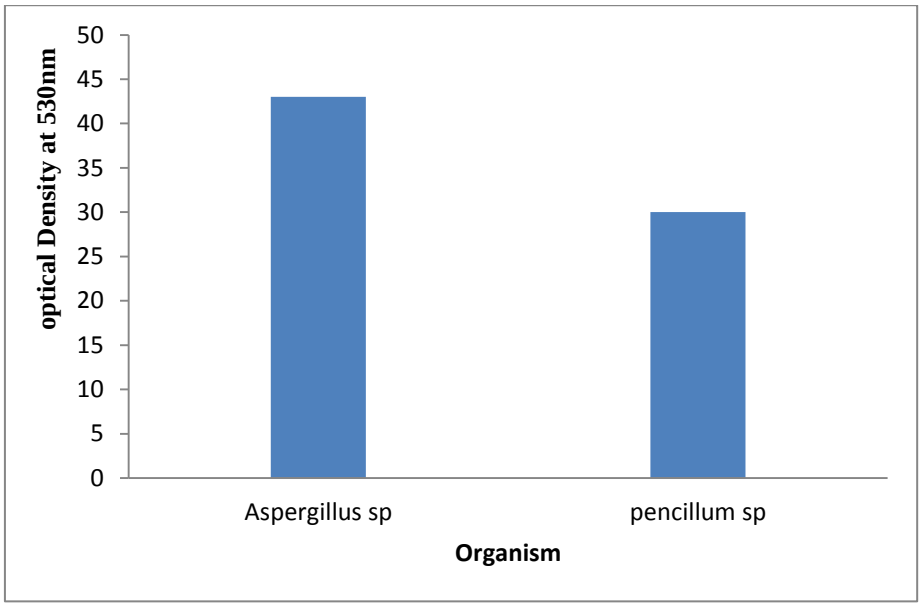

Figure 5: Amylase Activity

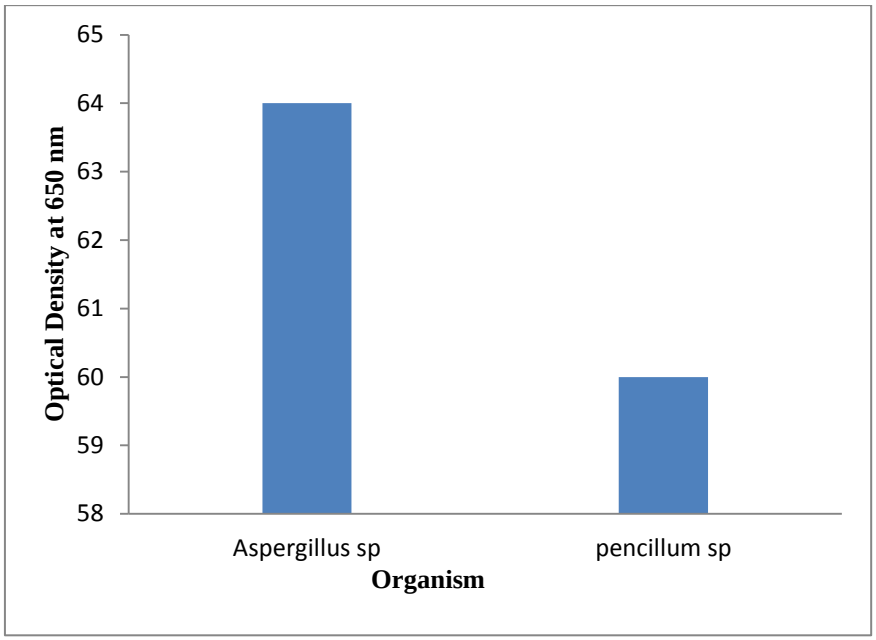

Figure 6: Protease Activity

identified. The R_f values were found to be 0.40,0.47 and 0.60 respectively for three amino acids. Based on the R_f values, the given amino acid was identified as Methionine, Tryptophan and Leucine by the standard R_f values. Sullivan et al (1971)[12] suggested that free amino acid is released in the culture filtrate due to the activity of proteolytic enzymes. The present study indicated comparatively *Aspergillus sp* was found to produce more amounts of amylase, protease and keratinase and the substrate decomposition was better with *Aspergillus sp.*

CONCLUSION

A number of microorganisms including different fungi are present in the environment. The feather and hair protein has been showed to be an excellent source of metabolizable protein and that microbial keratinase enhanced the digestibility of feather and hair keratin. These keratinolytic strains could be used to produce animal feed protein. In addition the selected isolates able to grow and display keratinolytic activity in keratin waste. It would also solve the waste disposal problem of poultry waste and limited resources recycling of keratinase waste would be beneficial financially and environmentally.

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