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Screening of Extracellular Keratinase Producing Fungi from Feather Processing Area in Shenbakkam, Vellore District, Tamilnadu, India.

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ABSTRACT

The aim of the study was to screen the extracellular keratinase producing fungi from the soil collected from feather processing area in Shenbakkam district of Vellore in Tamil Nadu, India. The isolation was performed by serial dilution and plating method of the collected soil sample. A total of two fungi were isolated from the collected soil sample and were considered for further study. The isolates were identified as *Mucor* spp. and *Aspergillus niger* by cultural and microscopic observations. The specific activity carried out by *Mucor* was 0.8 IU/mg in crude extract, 0.9 IU/mg in Ammonium sulfate precipitate and 0.94 IU/mg in dialysis. Similarly, the specific activity carried out for *Aspergillus niger* was 0.66 IU/mg in crude extract, 0.72 IU/mg in Ammonium sulfate precipitate and 0.85 IU/mg in dialysis.

Keywords: Feathers degradation, Keratin, Keratinase, Enzymatic assay, Partial purification

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INTRODUCTION

Keratin is a protein derived from a Greek word means “for horn”. Keratin is an insoluble fibrous cytoskeletal structural protein found in hair, feathers, nails and horns. The monomer of keratin assembles to form intermediate filaments. Keratin is a polymer of type I and type II intermediate filaments found in Chordates whereas type V intermediate filament are found in Non-chordates. The keratin consist of α and β keratin. The α-keratin is found in hairs, nails and horns of mammals and the β-keratin is found in feathers, claws and beaks. The β-keratin contains β-pleated sheets present in the epidermal layer of the skin making it waterproof and preventing from desiccation. In addition to intra and intermolecular H-bonds, keratin has large amounts of sulfur containing amino acids like Cysteine which is required for the disulfide bridge and adds permanent strength and rigidity [1].

Every year more than 10,000-20,000 tons of feathers are produced from poultry farms which are not dumped properly and leads to environmental pollution [2]. Though different approaches like land filling, burning, treating as animal feed are done but still the feather wastes are not degraded properly and leads to the development of different pathogens. Traditionally, alkali hydrolysis and steam pressure cooking were used for feather degradation but it destroyed the amino acid and consumed high amount of energy [3]. Therefore, to reduce the environmental wastes and pollution, enzymatic biodegradation is an alternative method to degrade the feather wastes. The keratin as enzyme is produced by different microorganisms which degrade the keratin substrate.

Keratinase is a class of proteolytic enzyme that has the capacity of degrading keratin substrate. It is an extracellular enzyme which attacks the disulfide bond of keratin. Keratinase are involved in microbial conversion of keratinous wastes and has potential applications in leather, detergent industries and cosmetics for drug delivery through nails and degradation of keratinized skin [4]. Keratinase is produced by different microorganisms like bacteria, fungi, actinomycetes in alkali pH and thermophillic temperature. The microorganisms producing keratinase are keratinolytic bacteria and keratinolytic fungi. The bacteria strain produces enzyme that selectively degrade the β-keratin. Different bacteria like- *Bacillus licheniformis, Burkholderia, Chrysobacterium, Pseudomonas* and *Microbacterium sp.,* can able to degrade the keratin [5]. Feather hydrolates produced by keratinolytic bacteria has been used as additive for animal feed, organic fertilizers etc. Different Actinobacteria like *Streptomyces pactum, S.albus* and keratinolytic fungi like- *Aspergillus sp, Trichophyton sp, Rhizomucor sp* etc carry out the keratin degradation [6].

MATERIALS AND METHODS

Sample Collection

The soil sample was collected from the Shenbakkam area (12°55’28”N, 79°07’12”E) of Vellore, Tamil Nadu, India. This place has been the feather dumping area since last 10 yrs. The soil sample was collected from a depth of 5 to 6 cm depth with the help of sterile spatula. The collected sample was brought under sterile condition to the Bio-Science Lab I, VIT University, Vellore, TN, India for further processing (figure 1).

Figure 1: Collected Soil Sample

Isolation of micro organisms

Isolation of fungi was performed by serial dilution and plating method on Potato Dextrose Agar (PDA). About 1 gram of soil sample was taken and mixed properly in 10 mL of sterilized distilled water and serially diluted to the range of 10^{-6}. A volume of 0.1Ml of diluted sample was inoculated on PDA plates from 10^{-5} and 10^{-6} dilutions respectively. The sample was uniformly spread by using L-glass rod. The plates were incubated at 28°C for 48-76 hours. The fungal isolates were further sub cultured on PDA media to obtain pure culture and stored at 4°C in refrigerator for further study.

Screening of Keratinolytic Fungi

Preparation of Keratin

The keratin substrate was prepared by the method of Wawrakiewiaz [7] .The chicken feathers (10gm) were mixed with 500 mL of DMSO (Dimethyl Sulfoxide) and heated in a hot air oven at 100°C for 2 hours. The soluble keratin was filtered and precipitated by adding cold acetone. The formed precipitate was centrifuged at 8000 rpm for 20 minutes. The resulting precipitate was washed by distilled water.

Fermentation Medium

The keratin substrate was incorporated in the production medium. The loop full of the isolated fungal culture was inoculated to 50 mL production medium containing (g/L) yeast extract 0.1, $MgSO_4.7H_2O$ 0.2, K_2PO_4 0.3, KH_2PO_4 0.4, $CaCl_2$ 0.22 [7]. The flasks were loaded on rotary shaker incubator at 2000 rpm at 28°C for 48-76 hours.

The keratinase production was carried out by submerged fermentation. After incubation, the production broth was transferred for centrifugation at 7000 rpm for 20 minutes in a cooling centrifuge. After centrifugation, the supernatant was collected which was the crude enzyme for further enzymatic activity.

Keratinolytic Activity Assay

The keratinolytic activity was carried out by a modified method of Letourneau [8] About 1 ml of reaction mixture contains 30 mg of keratin with 50 mM of TrisHCl Buffer with pH 8. A volume of 1 ml of protein sample was added to reaction mixture and reaction takes place at 120 rpm for one and half hour at 30°C. The tubes are placed in the ice cubes to stop the reaction and centrifuged at 10,000 rpm for 20 minutes. The amino acid liberated was measured at absorbance 600 nm by using spectrophotometer. The same enzymatic assay was carried out without sample under same conditions and used as blank.

Estimation of Protein Content

The total protein content was calculated by Lowry's Method [9]. Bovine Serum Albumin (BSA) was used as standard.

Partial Purification of Keratinase Enzyme

Partial purification of keratinase enzyme was done by Ammonium Sulphate Precipitation followed by dialysis [10]. About 20 ml of cell free extract was dissolved with ammonium sulphate to a saturation of 80%. The precipitate content was incubated in refrigerator for 24 hrs. The precipitate was centrifuged at 5000 rpm for 15 minutes. The pellets were stored in buffer for further study.

The enzyme mixture containing one sample was transferred to a dialysis bag and immersed in TrisHCl buffer at 4°C for 24 hrs. The buffer was continuously stirred by the help of a magnetic stirrer. The buffer was changed 2 to 3 times to obtain proper purification. Similar process was followed for the second sample respectively.

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Application

The degrading activity of keratinase producing fungi was determined by the rate of degradation of feathers containing keratin. For this, 100 mL of Potato Dextrose Broth (PDB) was prepared and sterilized. The chicken feathers were dipped into the test tubes containing PDB and the isolated fungi were inoculated to the tubes respectively. A tube without culture was kept as a control (figure 2). The tubes were incubated at 28^{o}C in a rotary shaker for 15 to 20 days.

After 15 to 20 days, it was observed that the feathers in the tubes were degraded by forming mucoid like substances around the feathers (figure3). This was due to the keratinase enzyme produced from the fungi which helps in the degradation of feathers.

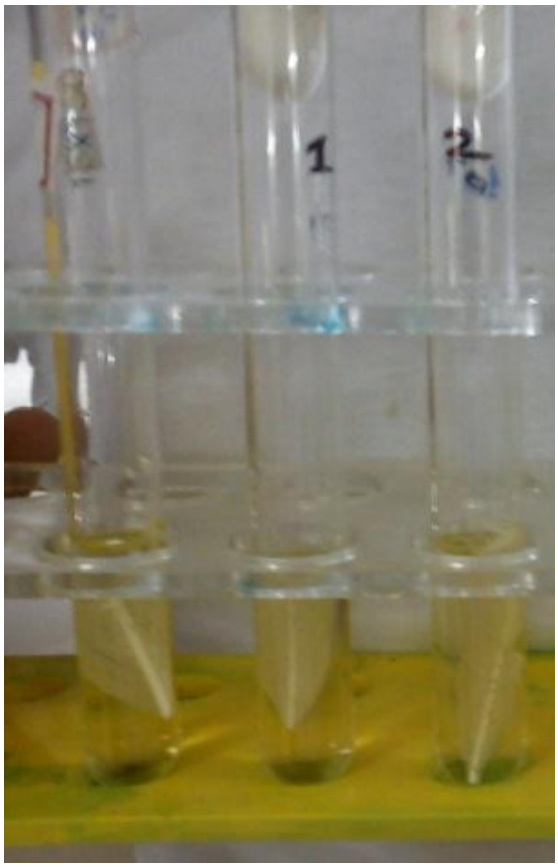

Figure 2: Feathers before the degradation

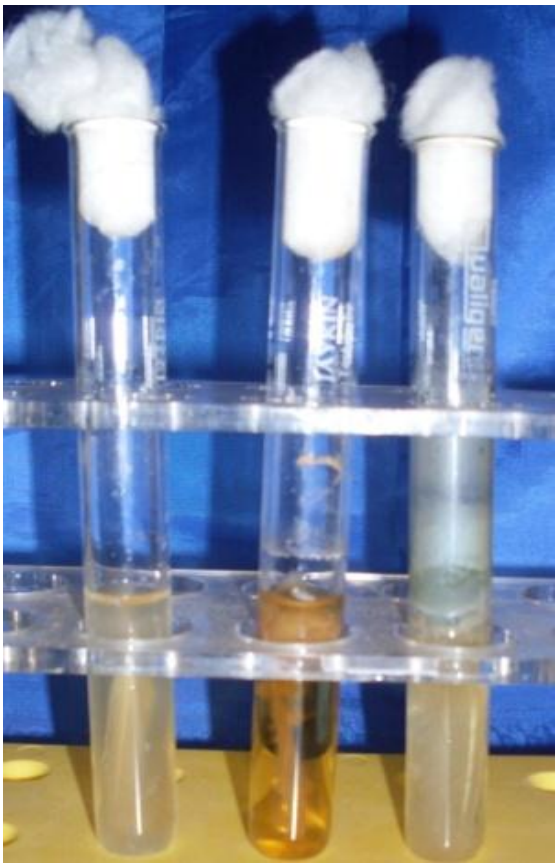

Figure 3: Feathers showing degradation.

RESULTS AND DISCUSSION

Isolation of keratinolytic fungi

A total of two fungi were isolated from the soil sample collected and were selected for further study of keratinase production and enzymatic activity.

Identification of keratinolytic fungi

For identify ication of the keratinolytic fungi, LPCB (Lacto Phenol Cotton Blue) mounting was performed. The fungi were identified as *Mucor sp* (figure: 4) and *Aspergillus niger* (figure: 5).

As per review of previous papers, different *Aspergillus sp* were isolated which showed keratin degradation. As compared to that, the fungi *Aspergillus niger* showed less degradation with less specific activity. Moreover, as per literature review, *Mucor* showed a high specific activity.

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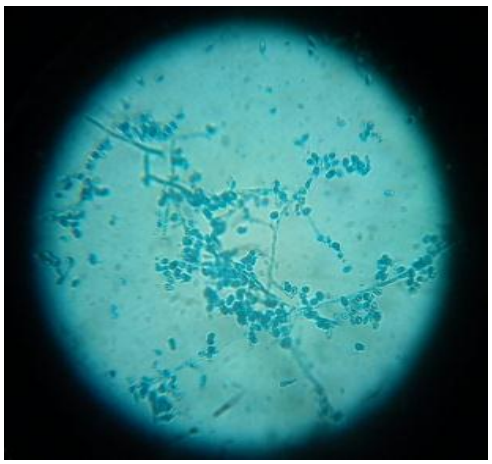

Figure 4: *Mucor* sp.

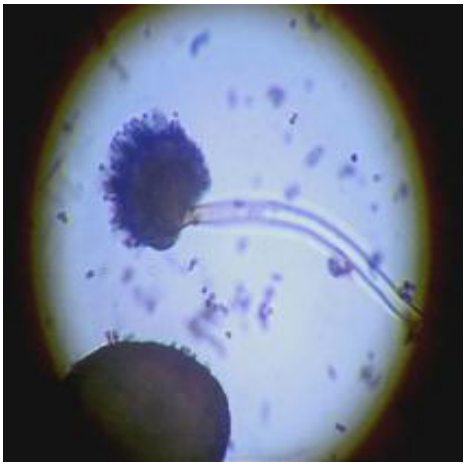

Figure 5: *Aspergillus niger*

Partial purification of keratinase

Partial purification of keratinase was done by Ammonium Sulphate precipitation followed by dialysis. The partially purified keratinase exhibited specific activity of 0.94 U/ml/µg which corresponds to 1.18 purification fold and 56.66% yield from *Mucor* sp. (Table 1) and again specific activity of 0.85U/ml/µg which corresponds to 1.28 purification fold and 46.7% yield from *Aspergillus niger* (Table 2).

Table 1: Partial Purification of Keratinase from *Mucor*

Purification Steps	Total protein (mg/mL)	Enzyme Activity (U/mL)	Specific Activity (U/mg)	Fold Purification	Yield %
Crude Extract	30	24	0.8	1	100
Ammonium Precipitate	20	18	0.9	1.12	66.66
Dialysis	17	16	0.94	1.18	56.66

Table 2: Partial Purification of Keratinase from *Aspergillus niger*

Purification Steps	Total protein (mg/mL)	Enzyme Activity (U/mL)	Specific Activity (U/mg)	Fold Purification	Yield %
Crude Extract	15	10	0.66	1	100
Ammonium precipitate	11	8	0.72	1.09	73
Dialysis	7	6	0.85	1.28	46.7

Keratin is a strong protein found in hairs, nails, feathers etc which is difficult to dissolve [11]. This strong property is due to the presence of cysteine that forms disulfide bridgeand creates a helix shape [12]. Different microorganisms like-actinobacteria, fungi, bacteria carry out the keratinolytic activity by the production of keratinase enzyme [13]. In the recent study, the fungi isolated from the soil sample collected from the Shenbakkam area of Vellore, TN, India undergoes the keratinolytic activity and the isolates were *Mucor* and *Aspergillus niger*. In the recent study, it has been reported that the *Mucor* sp. has high potential of keratinolytic activity as compared to *Aspergillus niger.* As per the review of previous literature, *Aspergillus sp.* grows on chicken feathers and degrades it releasing sulphydrlyl containing cysteine keratinase [14]. Moreover, keratinolysis is due to the ability of fungi to release soluble sulfur containing amino acids and polypeptides.

CONCLUSION

The present study reveals that, the fungal isolates are having moderate keratinolytic activity. In future, the enzyme activity may be increased by purification and media optimization. The fungal keratinase can be applied for feather degradation, wool cleaning or dehairing for leather industry and leads to have a pollution free environment.

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