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Evaluation of Keratinolytic Activity Succeeds by Keratinophilic Fungi in Jaipur, India

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Abstract: Earth has innate background for fungi that cover individual kingdom since evolution. The keratinophilic fungi are allied moulds that produce the keratinase enzyme to degrade the keratinous materials in or on the soil. Keratinous materials are insoluble and resistant to degradation by common proteinase enzymes. It is important to study the microorganism producers of such enzymes for use in the biotechnology industry. In order to present study, two isolates of fungi were evaluated to determine if they had the ability to degrade keratin as nutrient substrate. They were grown in an inundated culture medium containing poultry feathers. Among species, best keratin substrate degradation activity as well as keratinase enzyme activity was recorded in *Arthoderma multifidium* (KU578107) followed by *Chrysosporium tropicum* (KU578108) gradually leading manner day by days.

Keywords: *Arthoderma multifidium*, *Chrysosporium tropicum*, Enzyme, Filamentous Fungi, Keratin

Introduction

In poultry processing industry with market demand of contemptible meat, the feathers wastes are gradually increasing day by day. Especially in India, around 350 million tons per year poultry waste is produced from processing industries that are discarded or used for land filling and burned or buried (Agrahari and Wadhwa, 2010; Agrawal and Dalal, 2015; Kumawat *et al.,* 2016). The feather constitutes by β-keratin, a fibrous protein that highly cross-linked with disulfide bonds and appeared initial in 3-dimensional folds (Brandelli *et al*., 2010; Sharma *et al*., 2015a). These keratin wastes can be proficiently degraded by precise proteases such as keratinase. Keratinase is proteases which able to degrade the scleroprotein keratin that is produced by a domain of saprophytic and dermatophytes fungi, actinomycetes and other microbial species (Selvam and Vishnupriya, 2012; Sharma *et al*., 2015b). These groups contain a complex group of hydrolytic enzymes that disgrace to proteins into small amino acids. These hydrolytic enzymes attack on the carbonyl carbon of the scissile bond where the peptide bonds are cleaved by catalysis in addition of water (Bhat, 2000; Kumawat *et al*., 2013). Therefore, this present study was aimed to evaluate the degradation of keratin from feather degradation medium and to estimate a potential use of keratinase from keratinocytes fungi origin for the industrial treatment of keratin containing materials.

Materials and Methods

Soil Collection

The collection of soil samples was based on higher contamination of keratin at Jaipur. Soil samples were collected from the open roadside, public park, poultry farm house, slaughter house and barbershop dump area. The soil samples were taken by sterile spatula from the surface part and the depth was not exceeding 4-5 cm.

Isolation of Keratinophilic Fungi

The baiting technique of Vanbreuseghem was employed to isolate keratinophilic fungi from soil samples (Vanbreuseghem, 1952; Kumar *et al*., 2013). Sixty grams of soil samples were transferred into 90 mm sterile petri dishes and then the small piece of keratinous substances were aseptically spread on top of the soil sample. After that, the sterile distilled water (15-18 mL) was poured on the keratinous substrate baited plates. The baited plates were incubated at 27+2°C under low light for 21-25 days. The fungal/mold growths were appeared on the all keratinous substrates baited plates after 21-25 days. After that, the

fungal growths were culture and transferred on the slants of potato dextrose agar (Hi-Media) for pure culture isolation, identification and future analysis.

Identification of Isolated Fungi

After the preliminary examination, fungal growth was identified on the basis of macroscopic, microscopic and 18S rRNA sequencing. The Sequence was then submitted to NCBI Genbank.

(http://www.ncbi.nlm.nih.gov/Genbank/index.html).

Screen out the Keratinolytic Potential of Identified Keratinophilic Fungi

Fungi produce proteases a group of proteinases and peptidases with varying itself nature basis on species (Chaturvedi *et al*., 2013; Anand *et al*., 1990). In the present study, the keratin degradation by keratinophilic fungal species (*Chrysosporium tropicum* and *Arthoderma multifidium*) was evaluated using modified feather degradation medium (K_2HPO_4-1.25 g; $MgSO_4.7H_2O$-0.025 g; $CaCL_2$-0.02 g; $FeSO_4.7H_2O$-0.015 g; $ZnSO_4.7H_2O$-0.005 g; pH 7.0±0.2) for estimation of keratinolytic potential. In the process, the spore suspensions of 15 days old culture of each fungal species were inoculated in sterilized flask containing 100 mL of the FDM and 250 mg of keratin substrate and incubated at 27±2°C on the orbital incubating shaker at 70 rpm for 15 days.

Keratinase Enzyme Activity of Keratinophilic Fungi

The specific classes of proteolytics also contain keratinase that catalyze the hydrolysis of keratin substrates (Awasthi and Kushwaha, 2011). After 3-4 days, with the growth of fungal mycelium, there was started the consumption of keratin substrate and release an extracellular enzyme known as keratinase. In the mid of inoculation, on 4, 8, 12 and 16 day, After respective day's incubation, mycelium was removed by filtration and the filtrated was centrifuged at 10000 rpm using cooling centrifuged for 10 min and the supernatant was used as a crude enzyme (Riffel *et al*., 2003; Kim, 2003). Keratinase activity was evaluated by the modified method of Yu *et al*. (1968). In the method, 20 mg of chicken feathers powder was suspended in 3.8 mL of 100 nm Tris-HCL buffer with pH 7.8. About 200 µL of the centrifuged enzyme filtrate was added. The mixture was incubated to process keratinolytic reaction at 37°C for 1 h. Then the mixture was dipped into ice cold water for 10 min for shutting down the keratinolytic reaction. Finally, the mixture was filtered to remove a residue of remain feathers. Then the clear mixture was measured at 280 nm for absorbance by GE Healthcare Ultrospec™ 8000 Dual-beam UV-Visible Spectrophotometer. The keratinase activity was measured and expressed as one unit of the enzyme corresponding to an increase absorbance value 0.1 (1 KU = 0.100 corrected absorbance), KU = keratinase unit (Jaroslava *et al*., 2014).

Results

Isolation and Identification

Soil samples from Jaipur were enriched in feather-degrading microorganisms. On the basis of initial baiting keratinolytic screening, isolates were selected for further identification. As per the cultural, microscopic and molecular identification, these fungal species were identified as *Arthoderma multifidium* (KU578107) and *Chrysosporium tropicum* (KU578108).

Keratinolytic Potential of Identified Fungi

After keratin degradation feather degrading media, the residual feathers were harvested from the fermentation media by filtering it through Whatman filter paper.

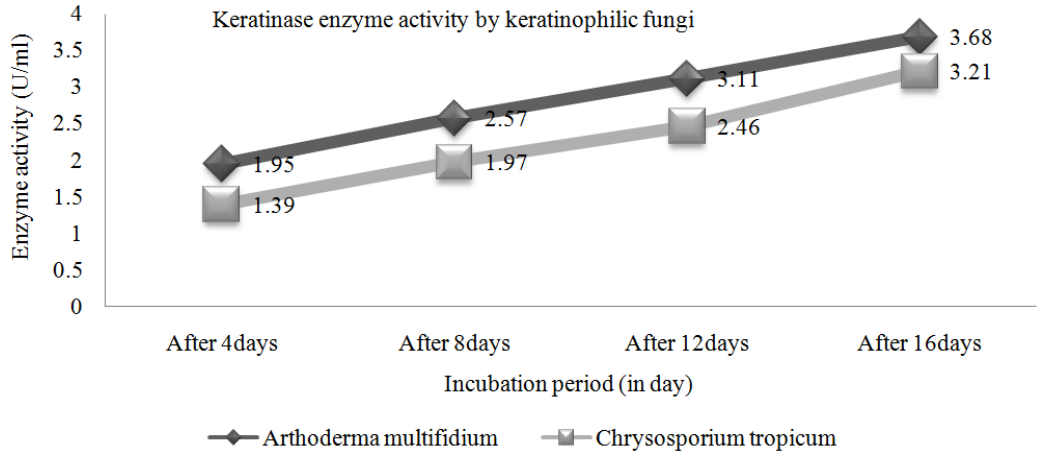

Fig. 1. Graphical representation of keratinase enzyme activity

Table 1. Final pH and the keratin degradation potential for each sample

Sample name	Final pH of medium	Keratin degradation potential (In +&-)
Arthoderma multifidum	7.42	++
Chrysosporium tropicum	7.39	+

- No inhibition; + Fair inhibition; ++ Excellent inhibition

A final pH of the culture filtrates was determined by calibrated pH meter. The presence of keratinous feather degradation was calculated in the positive or negative mode to verify the keratinolytic nature of selected species indicating with + for positive and - for negative (Table 1). Among the keratinophilic fungal species, best keratin substrate degradation activity was recorded in *Arthoderma multifidium* followed by *Chrysosporium tropicum*. The alkalinity of the medium was changed possibly due to the production of cysteine, keratinase and proteins. Observation was also showed that the fungi, although deamination and alkalization of the medium due to excretion of excess nitrogen via deamination and ammonium excretion, surely engage in the activity of keratinolysis (Fig. 1). Possibly in degradation, the disulphide linkage involves rupturing between the peptide chains of keratin molecules by some extra and intra cellular enzymes collectively called keratinizing activity (Buxman, 1981). In keratinase enzyme activity, both species were found positive for protease activity.

Subsequently in evaluation of keratinase enzyme activity by dermatophytic fungi, the keratinase enzyme activity was started in an increasing pattern during incubation period. In the present study, the best activity was recorded on 16 days of incubation in a gradually rising manner from 4th day of incubation. Among the same, the highest keratinolytic activity was recorded in *Arthoderma multifidium* (3.68 U mL^{-1}) with followed by *Chrysosporium tropicum* (3.21 U mL^{-1}). These extracellular enzymes secreted by these fungi are responsible for the degradation of keratin in nature.

Discussion

Enzymes are the catalytic keystone of metabolic actions of living organisms. Enzymes play a role as proteins with possessing its properties as particular towards the reactions that they catalyze and also as substrates on which they function upon (Bhat, 2000). The Fungal extracellular enzymes help to break keratin's macromolecules into micro molecules which they can absorb. For it, they require carbon substance as a source of energy and nitrogen substance to build protein and another essential compound. Further, a group of enzymes can be secreted simultaneously to target multiple nutrients (Chaturvedi *et al*., 2013; Anand *et al*., 1990). Keratinase is produced by various bacteria, actinomycetes and fungi in an optimum range of physical parameters (Farag and Hassan, 2004; Thys *et al*., 2004; Anbu *et al*., 2005). In the presented study, our study reports similar to Sousa *et al*. (2015; Jaroslava *et al*., (2014). In subsequence, Kushwaha and Agarwal (1976) isolated the same species from the Sagar, Madhya Pradesh, India. In Jaipur, a study was reported on the presence of *Chrysosporium tropicum* from soil samples of public park by Sharma and Sharma (2009). At Andhra Pradesh, India, Ramakrishnaiah *et al.* (2013) isolated five types of indigenous fungi from the decaying poultry feather samples and their enzymatic activity. In this study, feather degradation was determined visually. According to the graph, it is confirmed that *Arthoderma multifidum* and *Chrysosporium tropicum* fungi are also possessed a protease proficient of reducing disulfide bonds of keratin. Further optimization of the process needs to be done to make it suitable for commercial use. This includes optimization of feather concentration with the fungi directly as well as the isolated enzyme.

Conclusion

In Environment, the keratinase enzymes from keratinolytic organisms have used in animal feed processing, sewage treatment and even environmental bioremediation. In this study, both species (*Arthoderma multifidum* and *Chrysosporium tropicum* are a potential keratinolytic fungi which are suitable for the degradation of natural keratin wastes. There is a need to carry out further studies on these isolates to facilitate commercialization uses as catalysts in biotechnological applications involving bioremediation and hydrolytic reactions with an established degradation of the chicken feathers and other keratin containing wastes in environment.

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Author's Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of

the other authors have read and approved the manuscript and no ethical issues involved.

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