

ORIGINAL RESEARCH ARTICLE

Keratinase Activity of Some Hyphomycetous Fungi from Dropped Off Chicken Feathers

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ABSTRACT

In this study identified hyphomycetous fungi isolated from dropped off chicken feathers, showed keratinolytic ability by making a clear zone around their colonies on SMA medium. Among 101 keratinolytic hyphomycetous fungi, 13 species belonging to six genera examined for keratinase activity in submerged cultures using chicken feather as keratinous substrate. The highest keratinolytic activities were recorded in *Acremonium brunnescens* MTCC 10376, ($65.73 \pm$ ku/ml), *Chrysosporium indicum* MTCC 10377 (63.5 ± 2.47 ku/ml), *Acremonium chrysogenum* NFCCI 1883 (45.11 ± 1.59 ku/ml), *Acremonium byssoides* MTCC 9985 (41.66 ± 0.75 ku/ml), *Scopulariopsis stercoraria* NFCCI 1885 (34.6 ± 3.69 ku/ml) *Chrysosporium tropicum* NFCCI 1884 (24.69 ± 2.11 ku/ml), *Fusarium culmorum* GPCCK 3204 (22.91 ± 0.86 ku/ml) and *Alternaria alternata* NFCCI 1878 (20.8 ± 3.69). These novel nondermatophytic keratinolytic fungi have potential use in biotechnological processes involving keratin hydrolysis. The result of this work contributes to show that keratinolytic activity is relatively widespread among common hyphomycetous fungi and may have an important role in feather degradation in natural environment.

Key words: Feather waste, Keratin, Keratinase activity, Hyphomycetous

INTRODUCTION

The keratinous waste largely increasing and accumulating in the environment mainly in the form of feathers generated from poultry processing industries. Today it is also becoming a part of solid waste management since it is tough to degrade due to the highly rigid structure rendered by extensive disulphide bonds and cross linkage. Hence, there is a demand for developing biotechnological alternatives for recycling of such feather waste. Keratinase enzymes are widespread in nature and are elaborated by several microorganisms, most of them isolated from poultry waste. Keratinases in nature have been continuously contributing to valorization of voluminous keratin containing wastes in the form of hair, feathers, dead birds and animals [1,2]. Despite the recalcitrance, keratin waste can be efficiently degraded by a myriad of bacteria, actinomycetes and fungi due to the elaboration of keratinolytic proteases called “keratinases” [1]. Keratinolytic activity has been reported in many actinomycetes [3], bacteria [4], dermatophytes and saprophytic fungi which secrete keratinolytic

enzymes. Among the fungi keratinases from dermatophytic fungi have long been well known due to their notorious pathogenic nature and these enzymes have only recently gained biotechnological importance. Their growing importance is mainly contributed to the isolation of keratinases from non-pathogenic microorganisms and their ability to degrade keratin into economically useful keratin product [1,5,6], nitrogenous fertilizers, biodegradable films, glues and foils [7,8]. A specific class of proteolytic includes keratinases which catalyse the hydrolysis of keratins. They are the key enzymes in fungal invasion of skin and skin formations [9] and have been mostly studied for dermatophytes such as *Trichophyton* [10,11] and *Microsporum* [12,13] as well as the yeast *Candida* [14,15] which also causes skin infections. Known keratinases are mainly reported from submerged cultures of dermatophytes on keratinous substrate [16-22]. However, the production of such enzymes was not exclusively associated with dermatophytes [23-26]. Several keratinolytic, non dermatophytic and dermatophytic fungi survive in the soil, in

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addition to their clinical habitat. Nondermatophytic fungi can also degrade keratin *in vitro* and produce proteolytic enzymes including keratinase^[27,28]. The most keratinolytic group among fungi belongs to fungi imperfectii. Hyphomycetous fungi synthesize a variety of hydrolytic enzymes. Since the keratinases of many hyphomycetous fungi have not exploited it is therefore need of the day to exploit them. The mechanism of colonization and decomposition of the keratinous substrates have received little attention. The fungi which degrade these substrates completely are termed as keratinolytic. Keratinolytic enzymes are widespread in nature and are elaborated by a numerous microorganisms isolated from different habitats and sources. The accumulation of chicken feathers in natural setting suggests the existence of keratinolytic microorganisms. These feathers degrading hyphomycetous fungi may contribute to understanding the role of these fungi in the degradation of complex keratinous substrate in nature.

The aim of present study was to isolate and screen non dermatophytic fungi for their ability to produce keratinase in submerged and stationary cultures, using chicken feathers as the sole source of carbon and nitrogen.

MATERIALS AND METHODS

Isolation

The fungi were isolated mainly from soil of Kanpur city, U.P. For the screening of 101 hyphomycetous fungi of 13 genera were used. The classification is shown in (Table 1). Hyphomycetous includes *Acremonium* (17 strains), *Alternaria* (2 strains), *Aspergillus* (6 strains), *Chrysosporium* (32 strains), *Fusarium* (10 strains), *Malbranchea* (3 strains), *Mycelia sterilia* (4 strains), *Paecilomyces* (1 strain), *Penicillium* (1 strain), *Scopulariopsis* (1 strain) and *Trichoderma* (2 strains) and Dematiaceous hyphomycetous includes *Bipolaris* (1 strain), *Chaetomium* (1 strain), *Cladosporium* (18 strains) and *Curvularia* (2 strains). All cultures were deposited in DST sponsored Germ Plasm Centre for Keratinophilic Fungi (GPCK), Department of Botany, Christ Church College, Kanpur, Microbial Type Cultures Collection (MTCC), Chandigarh and National Fungal Culture Collection of India (NFCCI), ARI Pune.

Screening for their keratinolytic potential

The selected strains were screened by following two methods:

1. Measurement of transparent clearing zone on solid milk agar plates

The fungi were tested for their keratinolytic activity on solid skim milk agar plates using a modified medium^[29]. Solid skim milk agar plates were inoculated with 6 mm disc of 8 days old culture of selected fungi and incubated at 28±2°C for 8 days. The transparent clearing zone formation on solid milk agar plates and the diameter was measured to quantify the keratinolytic activity of fungus. All the tests were run in triplicates. The observations were taken at 8 days of incubation period and average was the result of keratinolytic activity of fungus.

2. Feather degradation in liquid medium

Feather degradation was assessed by measuring keratinase activity of selected fungus by the modified method^[30]. Fungal strains showing the highest activity on skim milk agar plates were tested further for keratinase activity. In order to confirm the previous screening, a chicken feather was selected as a keratin substrate to induce enzyme synthesis. Chicken feathers were purchased from local market and brought to the laboratory and washed with distilled water followed by air drying. These were weighed in to 200 mg aliquots. A basal medium was prepared some modifications of existed one^[30]. It contained the following ingredients per litre of distilled water: Glucose – 2 gm, Peptone – 5 gm, Yeast – 5 gm, K₂HPO₄ – 1 gm, KH₂PO₄ – 3 gm, CaCl₂ – 1 gm, MgSO₄ – 1 gm, Feather - 200 mg per flask. The 250 ml Erlenmeyer flasks containing 50 ml of the basal medium and 200 mg of chicken feathers as a keratin substrate were autoclaved at 15 lbs pressure for 10 minutes. The pH of the medium was adjusted to 7.0 before sterilization. Spore suspension of the fungal isolates was prepared by adding 10 ml of sterilized water to 8 days old fungal isolates growing on plates of potato dextrose agar. The final concentration of the spore suspension was adjusted to about 2x10⁶ mL⁻¹. The flasks were incubated in at 28±2°C. All the experiments were carried out in triplicates.

Enzymatic analysis

Keratinase activity was measured by following method^[30] with some modifications. 1 ml of culture filtrate, 4 ml of glycine / NaOH buffer (0.05 M/ pH 10) and 20 mg of feather was incubated at 40° C for 60 minutes. After 1 hour the reaction was terminated by adding 4 ml of 5 % of trichloro acetic acid and tubes were incubated for 60 minutes at room temperature. The feather and insoluble residues were removed by glass wool and the filtrates were centrifuged at 3000 g for 5 minutes. An enzyme control was prepared in a similar manner, except that 1 ml of trichloro

acetic acid and 3 ml of the buffer were added instead of 4 ml of the buffer used in the test samples. Keratolytic products in the supernatant were determined by reading absorbance at 280 nm against basal medium using UV-Spectrophotometer (SHIMADZU). An increase of 0.100 in the absorbance was considered as equivalent to 1 unit of KU (keratinase unit).

RESULTS AND DISCUSSION

Screening tests on skim milk agar showed that all the hyphomycetous fungi were able to grow in the given environmental conditions (**Table 1**). Hyphomycetes were exhibited growth and developed a clearing zone on skim milk agar by the excreted enzymes and showed keratinolytic activity. The clearing zone was measured at 8 days of incubation period. About 23.76% fungi showed 21-28 mm where as 58.41% showed 11-20 mm at 8 days of incubation period. Only 17.82 % showed 2-10 mm at 8 days. Some fungi capable to grow but did not clarify the medium, presumably due to the lack of extracellular enzymes. *Acremonium*, *Alternaria*, *Aspergillus*, *Chrysosporium*, *Cladosporium*, *Fusarium* and *Scopulariopsis* showed maximum keratinolytic ability on skim milk agar. The species of *Acremonium*, *Chrysosporium* and *Cladosporium* were specially keratinolytic in nature where as very low activity was found in the case of *Curvularia* in 8 days. Among the 101 filamentous fungal isolates screening, 13 proved to have keratinolytic activity. They were selected and tested for the production of keratinase in submerged cultures, are shown in (**Table 2**). The highest keratinase activity was found in culture filtrates of *Acremonium brunnescens* MTCC 10376, (65.73± 0.86 ku/ml), *Acremonium byssoides* MTCC 9985, (41.66±0.75 ku/ml), *Acremonium chrysogenum* NFCCI 1883, (45.11±1.59 ku/ml), *Chrysosporium indicum* MTCC 10377, (63.5±2.47 ku/ml) and *Scopulariopsis stercoraria* NFCCI 1885, (34.6±3.69 ku/ml), where as in *Chrysosporium tropicum* NFCCI 1884, (24.69±2.11 ku/ml), *Fusarium culmorum* GPCCK 3204, (22.91±0.86 ku/ml), *Alternaria alternata* NFCCI 1878, (20.8±3.69 Ku/ml), *Alternaria citri* GPCCK 3033, (19.35±0.61 ku/ml), *Cladosporium cladosporioides* MTCC 9983, (18.57±1.84 ku/ml) *Cladosporium herbarum* GPCCK 3030, (16.56±1.36 ku/ml), *Chrysosporium siglerae* GPCCK 3042, (15.62±0.95 ku/ml), *Cladosporium chlorocephalum* GPCCK 3069, (10.98±0.50 ku/ml) were also showed keratinase activity in their culture filtrates. Dermatophytes are the most

widespread group of hyphomycetous fungi, which can degrade keratin substrate completely and thus a great interest of scientists due to their pathological importance. However, the isolation of non-dermatophytic hyphomycetous fungi that efficiently degrade feathers is interesting because of these fungi play a significant role in the keratin degradation in natural setting. Most of the keratinolytic fungi are belonging to fungi imperfecti^[1,2]. In this respect, among 106 filamentous fungi isolated from poultry farm waste, 13 species belonging to seven genera (*Aspergillus*, *Acremonium*, *Alternaria*, *Beauveria*, *Curvularia*, *Paecilomyces* and *Penicillium*) were able to grow and produce keratinase in stationary cultures using poultry feather powder as the only substrate^[26]. The fungi we studied were able to grow normally, using chicken feathers as their sole source of carbon and nitrogen. The results showed that insoluble non-degradable chicken feathers were gradually decreased with the time, presumably due to keratin hydrolysis by keratinase of these hyphomycetous fungi. The maximum keratinase activity was found in *Acremonium brunnescens* (MTCC 10376) and *Chrysosporium indicum* (MTCC 10377) where as low activity was recorded in *Cladosporium chlorocephalum* (GPCCK 3069). 300 common fungi were screened for synthesis of extracellular keratinase, about 54% of the fungi grew on agar plates with soluble keratin and excreted the enzyme^[31]. Some representatives of *Fusarium*, *Acremonium* and *Geotrichum* were the most active and others (*A. flavus*, *Alt. radicina*, *Trichurus spiralis* and *Stachybotrys atra*) proved to be powerful producer of extracellular keratinases when cultivated in submerged conditions in a medium with porcine nail as the sole source of carbon and nitrogen.

Some species of fungi are already known to colonize feathers or wool and some, such as *Alt. alternata* and *Alt. tenuissima*, *A. flavus*, *Stachybotrys chartarum* and *Trichurus spiralis*, are known as protease producers^[32]. The use of microbial keratinases to upgrade the nutritional value of feather meal has been described^[33]. In this regard, keratinolytic enzyme may have important uses in biotechnological processes involving keratin containing wastes from poultry and leather industries, through the development of non-polluting processes^[34]. Besides their use in traditional industrial sectors like detergent, medicine, cosmetics, leather and feed, application in newer fields like prion degradation for treatment of the dreaded madcow disease,

biodegradable plastic manufacture and feather meal production can be called “modern proteases”^[2]. Although many applications of keratinases are still in the stage of infancy, a few have found way to commercialization, particularly the use of Bioresource International’s (BRI) Versazyme for feather meal production. Despite the availability of several reports, keratinolytic microorganisms are increasing day by day, there is only one extensive review ^[1] available on keratinases that emphasizes their biotechnological potential with respect to feather meal production.

Further, the mechanism of keratinolysis is highly complex and not yet well understood.

The possibility of using considered hyphomycetous fungi for biotechnological purpose is because of their cosmopolitan nature. Our results indicated that, *Acremonium* and *Chrysosporium* are well known genera and the keratinolytic activity is relatively common in hyphomycetous fungi. The identification of hyphomycetous species able to degrade keratin may help us to understand the role of these fungi in the degradation of complex keratinous substrates in natural setting.

Table.1. general classification of the tested fungal strains for their keratinolytic ability

Class	No. of genera	No. of species	Genera
Hyphomycetous	10	31	<i>Acremonium</i> , <i>Aspergillus</i> , <i>Chrysosporium</i> , <i>Fusarium</i> , <i>Malbranchea</i> , <i>Mycelia</i> , <i>Penicillium</i> , <i>Paecilomyces</i> , <i>Scopulariopsis</i> , <i>Trichoderma</i>
Dematiaceious hyphomycetous	5	10	<i>Alternaria</i> , <i>Bipolaris</i> , <i>Chaetomium</i> , <i>Cladosporium</i> , <i>Curvularia</i>

Table.2. Keratinase activities of some fungi isolated from dropped feather

Fungal species	Keratinase activity (Ku/ml)
<i>Acremonium brunnescens</i> (MTCC 10376)	65.73± 0.86
<i>Acremonium byssoides</i> (MTCC 9985)	41.66±0.75
<i>Acremonium chrysogenum</i> (NFCCI 1883)	45.11±1.59
<i>Alternaria alternata</i> (NFCCI 1878)	20.8±3.69
<i>Alternaria citri</i> (GPCK 3033)	19.35±0.61
<i>Chrysosporium indicum</i> (MTCC 10377)	63.5±2.47
<i>Chrysosporium siglerae</i> (GPCK 3042)	15.62±0.95
<i>Chrysosporium tropicum</i> (NFCCI 1884)	24.69±2.11
<i>Cladosporium chlorocephalum</i> (GPCK 3069)	10.98±0.50
<i>Cladosporium cladosporioides</i> (MTCC 9983)	18.57±1.84
<i>Cladosporium herbarum</i> (GPCK 3030)	16.56±1.36
<i>Fusarium culmorum</i> (GPCK 3204)	22.91±0.86
<i>Scopulariopsis stercoraria</i> (NFCCI 1885)	34.6±3.69

Each value represents mean ± SD of three independent determinations.

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