

Full Length Research Paper

Keratinolytic activity exhibited in an indigenous non-dermatophytic fungus strain *Cochliobolus lunatus* isolated from Khairpur Sindh Pakistan

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Keratins are the widely distributed fibrous proteins of our environment. Keratinase are proteolytic enzymes responsible for hydrolyzing insoluble keratin largely produced by microorganisms including fungi. The isolation, identification and characterization of a non-dermatophytic keratinolytic fungal strain of *Cochliobolus lunatus* from the soils of Khairpur Sindh Pakistan were reported. The temperature optimum for this strain production of the enzyme was 30°C, preferable carbon and nitrogen sources were glucose and gelatin respectively, optimum time for keratinase production was 9 days and the activity was suppressed by Ca²⁺ ions.

Key words: Keratinase, fungus, identification, characterization.

INTRODUCTION

Keratins are the widely distributed fibrous proteins of our environment found in epithelial cells of vertebrates and characterized by its high content of cystine and serine arginine amino acids. It is present in hair, feather, hooves, wool, horns, nail and *stratum corneum* (Sharma and Prashar, 1997).

Keratins, due to the presence of the disulfide linkages, coiled-coil in the structure, hydrophobic interactions, and hydrogen bonds, are highly resistant to acids and some protease enzymes, therefore, keratinous material is water insoluble and extremely resistant to degradation by proteolytic enzymes such as trypsin, pepsin, and papain, however, are easily digested by alkalis and keratinase enzymes (Williams et al., 1990; Fuchs, 1995).

A group of secreted proteolytic enzymes, which are able to hydrolyze insoluble keratins more efficiently than other proteases, are called keratinases (EC 3.4.99) (Onifade, 1998). They are produced by some insects and mostly by microorganisms. There are several microorganisms such as bacteria, actinomycetes and fungi that produce keratinase enzymes responsible for keratinolysis.

Several dermatophytes and other keratinase enzyme producing organisms have been found to exist saprophytically in soil (Abarca et al., 1989). In most soil studies, baiting techniques have been used to isolate keratinolytic microorganisms. The method which has been used most successfully is the hair bait technique and was first used for isolation of dermatophytes by Vanbreuseghem (1952). The prospective use of keratinases is in diverse applications where keratins is hydrolyzed, such as the leather and detergent industries, textiles, waste bioconversion, medicine, and cosmetics for drug delivery through nails and degradation of keratinized skin (Gupta and Ramnani, 2006). Proteolytic enzymes are largely used in the industry for biotechnological applications involving the hydrolysis of protein substrates. Proteases constitute an important fraction of the global enzyme sales, and a relevant part of this market is accounted by bacterial proteases (Rao et al., 1998). The aim of present study was to investigate the presence of keratinase producing fungi in the indigenous soil and report on some of their main biochemical and physiological properties. The presence and potentials of such microorganisms has not been studied so far and this is the first report of characterization of keratinase producing fungi from Khairpur Sindh Pakistan.

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Table 1. Composition of skimmed milk agar.

Nutrient	Quantity (%)
NaCl ₂ (Merck)	5
Peptone (Oxoid)	3
Agar (Oxoid)	2
Skimmed Milk	1
pH	7.0
Distilled water	100 ml

Table 2. Composition of basal salt feather medium.

Nutrient	Quantity (g/L)
NaCl ₂ (BioM Malaysia)	0.5
MgCl ₂ (Sigma)	0.1
CaCl ₂ (Sigma)	0.06
KH ₂ PO ₄ (Merck)	0.7
Feather Meal (prepared in this study)	10

MATERIALS AND METHODS

Isolation and growth conditions

One hundred and twenty five (125) soil samples were collected from different sites namely fertile soil, barber shops, animal slaughter house, poultry house and animal herds in District Khairpur. Soil samples (40 g) were collected from 25 to 30 mm depth using sterile tools; in the sterile polythene bags and brought to the research laboratory of the Department of Microbiology, Shah Abdul Latif University, Khairpur for the isolation of keratinase enzyme producing microorganisms. For the isolation of fungi, Hair Bait Technique (Vanbreuseghem, 1952) was used. Soil samples in duplicate were placed in sterilized Petri plates; moistened with 25% (w/v) sterilized water and baited by placing short filaments of autoclaved sterilized horse and human hair upon the surface of the soil. The Petri-plates were incubated at 29°C for one to two weeks, and examined for the development of mycelium on the hair filament. After the appearance of mycelia, a piece of that invaded hair was inoculated on the surface of the Sabouraud's Dextrose Agar (SDA, Oxoid) and Czapek Dox Agar (CDA, Oxoid) and incubated at 30°C. Growth was identified on the basis of their colonial and microscopic characteristics (presence of spores, mycelial fragmentation, aerial spore mass, color and production of soluble pigments).

Preparation of chicken feather powder

Chicken feathers collected from poultry farms of District Khairpur were washed with distilled water and dried overnight at 60°C as described by Guichard (2008). Dried feathers were ground into (Anex Germany Products GMBh) a fine powder.

Screening of fungi for keratinase production on skimmed milk agar

Skimmed milk (10 g, Nido) was suspended in 10 ml distilled water to give final concentration of 1% and autoclaved at 121°C for 6 min. The rest of the ingredients listed in Table 1 were dissolved in 90 ml 50 mM sodium phosphate buffer (pH 8) by heating; when it was fully homogenized, it was autoclaved at 121°C for 15 min. After cooling to 45°C, both were mixed and plated.

Submerged fermentation

The Submerged fermentation medium (Basal salt feather medium, Table 2) was prepared as described by Hoq et al., (2005). Conidia suspension of fungal spores containing 80 000 spores per ml (counted by Neubauer's chamber) was prepared in saline and incubated at 30°C for seven days in an orbital shaking incubator at 150 rpm.

Crude keratinase enzyme was collected by centrifugation of the culture at 6 000 rpm for 10 min at room temperature and filtered through an Acro-disc filter (0.2 µm membrane pore size) in the sterilized flask. By this process, the crude keratinase enzyme was prepared after which the assay was performed for enzyme activity.

Assay of keratinase activity

Keratin azure (Sigma-Aldrich, USA) was used as the substrate. It was first frozen at - 0°C and then ground into fine powder. The 5 mg keratin azure powder was suspended in 1 ml 50 mM Tris-HCl buffer (pH 8.0). The reaction mixture contained 1 ml keratin azure suspension and 1 ml crude enzyme. The control comprised a 1 ml keratin azure suspension and 1 ml basal salt feather medium. The reactions were carried out at 50°C in a water bath with constant agitation of 200 rpm for 30 min. After incubation, the reactions were stopped by adding 2 ml 0.4 M trichloroacetic acid (TCA) and followed by centrifuging at 3000xg for 5 min to remove the substrate. The supernatant was spectrophotometrically measured for the release of azo dye at 595 nm against control. One unit (U) keratinase activity was defined as the amount of enzyme causing 0.01 absorbance increase between the sample and control at 595 nm under given conditions.

Protein determination

Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin (BSA) (Sigma, USA) as standard. The specific activity was expressed as the enzymatic activity (U) per mg of protein.

RESULTS

A total of 125 soil samples (25 from each sampling site) were collected for isolation of keratinase producing fungi from Khairpur district Sindh Pakistan. The results of the isolation of keratinase producing fungi are presented in Figure 2. The isolation was carried out using Hair Bait Technique and inoculating invaded hair on growth medium (Figure 1). The data revealed that out of 125 fungal species, maximum numbers (85/253, 33.6%) was isolated from soil of fertile lands followed by the soil samples from poultry farms (68/253, 26.9%). Isolation rates of keratinase producing fungi from the soil of herds (44/253, 17.4%) and barber's shops (43/253, 17.0%) were almost similar.

The lowest number of these fungi was isolated from the slaughter house soils (13/253, 5.1%). A total of 253 isolates of keratinase producing fungi including eight genera and 11 species were isolated viz, *Aspergillus niger* (20.2%), *Alternaria alternata* (13.0%), *A. flavus* (11.9%), *A. fumigatus* (11.1%), *Botrytis cinaria* (18.2%), *Chochliobolus lunatus* (16.8%), *Mucor spp* (16.0%), *Chrysosporium asperatum* (9.9%), *Fusarium spp* (5.1%), *Penicillium spp*: (3.1%) and

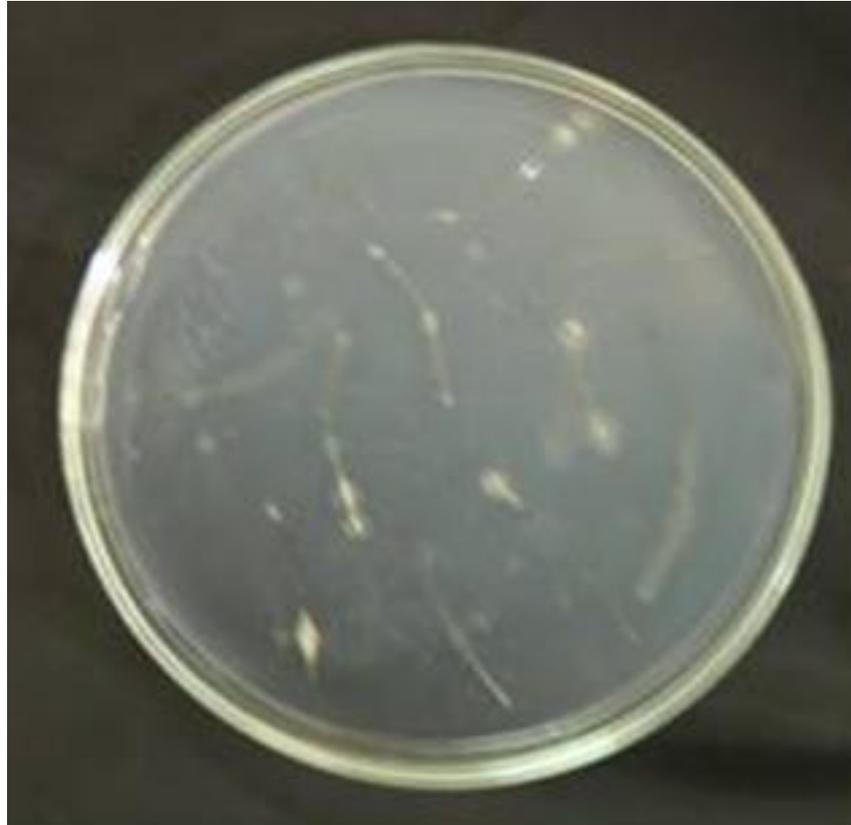


Figure 1. Invaded hair inoculation on growth media. An isolation of Keratinase producing fungi by direct hair inoculation on SDA. The growth was observed after 3 days of incubation at 30°C medium.

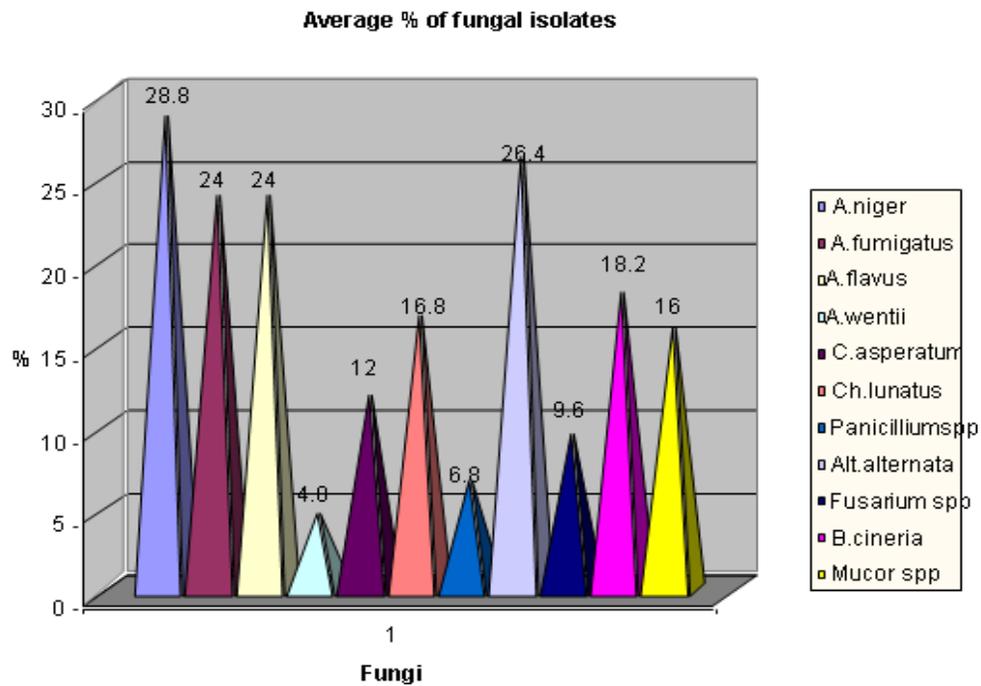


Figure 2. The Average percentage of isolation of fungal species from 125 soil sample collected from various sites using techniques described in the materials and methods. Total isolates were 253 (100%).

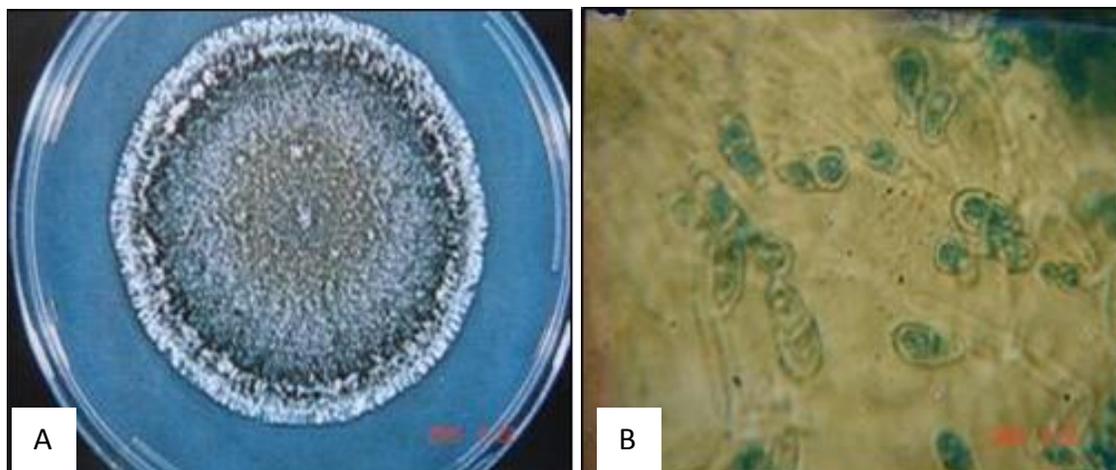


Figure 3. Isolation and Identification of Keratinolytic *Cochliobolus lunatus* by Hair Bait Technique (A) and Macroconidia (B) from different soil samples in Khairpur.

A. wentii (2.4%). The confirmation of the fungal species was performed on the bases of colony morphology, pigmentation, conidial characteristics and microscopy. Keratinolytic fungal species *C. lunatus* was isolated by hair bait technique and identified as described in materials and methods (Figure 3). The enzyme activity was screened using skimmed milk agar and expressed as diameter of clear zones. Among all the fungal species, *C. lunatus* showed the largest zone of hydrolysis at 30°C.

Effect of temperature

Effect of temperature ranging from 30 to 50°C on the growth and zone of hydrolysis was determined on skimmed milk agar. *C. lunatus* grown and produced 6 and 2 mm zones at 30, 35 and 40°C, respectively, where no zone was observed at 45°C. The organism stopped growth at 50°C.

Effect of carbon sources

The effect of different carbon sources on growth and enzyme activity of *C. lunatus* was observed after one week of incubation at 30°C. *C. lunatus* responded to carbon sources [Glucose, Maltose, Sucrose, Lactose and Cellulose (1% final concentration)] as assessed by zone on skimmed milk agar. The zone of hydrolysis was observed in medium containing glucose (6 mm zone size) maltose (5 mm zone size) and sucrose (2mm zone size) but no zone was observed around growth in the medium containing lactose and cellulose.

Effect of nitrogen source

Effect of nitrogen source (gelatin and peptone, 0.1% final concentration) was observed on the growth and proteolytic

activity of *C. lunatus* at 30°C after one week of incubation. Gelatin supported the growth and proteolytic activity of *C. lunatus* and produced zone of hydrolysis (4 mm) on skimmed milk agar, whereas, peptone did not support the growth and proteolytic activity.

Effect of metal ions

Effect of different metal ions (5.0 mM) on growth and proteolytic activity of keratinase production from *C. lunatus* was observed using skimmed milk agar as a medium. NH_4Cl , KH_2PO_4 , K_2HPO_4 , NaCl supported the growth and MgSO_4 and CaCl_2 suppressed the growth of the *C. lunatus*.

The activity was measured by keratin azure assay as described in material and methods. *C. lunatus* produced 161 U/mg enzyme activities on the 9th day of incubation time after which the activity declined (Figure 4).

DISCUSSION

This study presents the keratinase production from a fungal strain of *C. lunatus*, isolated from Khairpur district. The screening study revealed that, this fungus possesses keratinase activity. The isolation of keratinolytic saprophytic fungi has been reported before (Abarca et al., 1989). The best studied are keratinases from the dermatophytic genera *Microsporium* (Mukhopadhyay and Chandra, 1990); and *Trichophyton* (Tsuboi et al., 1989). Recently, keratinase producing *Aspergillus oryzae* was reported by Ali et al. (2011) with specific activity of 2312.7 U/mg. *C. lunatus* keratinase activity has not been previously reported although the isolation from goat and sheep hair has been described by El-said et al. (2009).

The production and characterization of keratinase from indigenous *C. lunatus* were reported. The enzyme activity

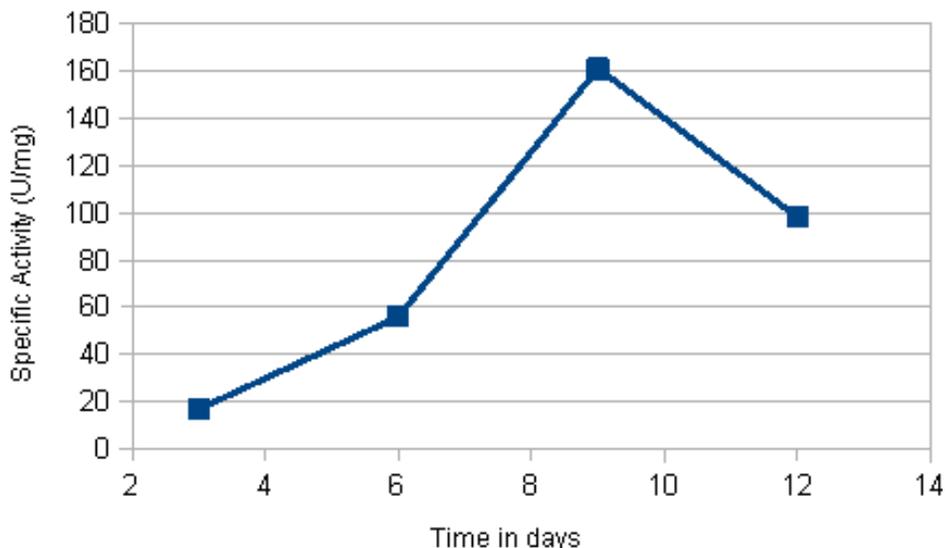


Figure 4. The enzyme activity measurement of keratinase from *C. lunatus* during growth from 1 to 9 days under submerged fermentation conditions as described in material and methods. Enzyme activity was measured after every 3 days.

was revealed by the zone of hydrolysis on skimmed milk agar. The 30 to 40°C temperature optima for *C. lunatus* placed this fungus in mesophilic category. Most of the other keratinases from fungi show optimal activity at roughly 40°C (Cheng et al., 1995; Gradisar et al., 2005; Santos et al., 1996). Different temperatures have been reported in the literature for optimal production and activity of keratinases by a variety of fungi, each showing specific temperature requirement (El-Naghy et al., 2001).

Further to this, the feather powder prepared in this study supported the growth of the fungus as a sole carbon and nitrogen source. This indicated the potential of the *C. lunatus* for utilizing keratin and production of keratinase. Specific enzyme activity of keratinase when measured using keratin azure as a substrate; show that the crude enzyme preparation contained keratinase activity. Time course study estimated the production of keratinase from *C. lunatus* at different growth intervals that showed maximum enzyme production on the 9th day of incubation. As being filamentous fungi, it is presumed that *C. lunatus* required longer time for growth and production of the metabolites. Considerably, lower activity was found in the medium containing sucrose where preferable carbon sources appeared to be glucose and maltose. No activity was observed with lactose and cellulose. This is in good agreement with the studies of Kaul and Sumbali (1999; 2000) and Naghy et al. (2001) but Santos et al. (1996) reported that the presence of glucose in the medium decreased the keratinolytic activity of *Aspergillus fumigatus* cultures and this disagrees with our report. These observations suggest that, response to sugars varies across different fungal species. Thus, detailed studies should be performed for each species and system of interest.

As a nitrogen source, gelatin supported the growth and enzyme activity of *C. lunatus*. This is in good agreement with Malviya et al. (1992) and Chopra and Mehta (1985) who reported maximum proteinase production with gelatin which served as poor substrate for fungal growth. The utilization of peptone was not favored due to the influence of peptone on the osmotic pressure. Suppressed protease activity as assessed by zone of hydrolysis in presence of CaCl_2 indicated that the enzyme was not a metalloprotease. It has been reported that Calcium ions activated keratinase activity at lower concentrations whereas at higher concentrations; the activity was suppressed (Yu et al., 1969). Similar results have also been reported by Tork et al. (2010) where slight reduction in the presence of CaCl_2 was found. Thus, presumably, the keratinase from *C. lunatus* belongs to serine protease family.

Conclusion

A non-dermatophytic keratinolytic fungal strain of *C. lunatus* was isolated, identified and characterized from soils of Khairpur. The isolation, identification and characterization of a non-dermatophyte keratinolytic fungi shows that besides being a plant pathogen, this fungus strain exhibits keratinolytic properties and can utilize keratin for its growth. Thus, this strain is keratinophilic/keratinolytic in nature and may play a significant role in biodegradation of keratin substrate.

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