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

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Abstract: 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. We report the isolation, identification and characterization of a non-dermatophytic keratinolytic fungal strain of *C. lunatus* from the soils of Khairpur Sindh Pakistan. The temperature optimum for this strain for production of the enzyme was 30^0C, preferable carbon and nitrogen sources were glucose and gelatin respectively; optimum time for keratinase production was 9 days and the activity suppressed by Ca suggesting it a metalloprotease.

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INTRODUCTION

Keratins are the widely distributed fibrous proteins of our environment found in epithelial cells of vertebrates and characterized by its high content of amino acids, especially cystine, arginine and serine. It is present in hair, feather, hooves, wool, horns, nail, and stratum cornium[1]. Keratins, due to the presence of the disulfide linkages 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[2,3].

A group of proteolytic enzymes which are able to hydrolyze insoluble keratins more efficiently than other proteases are called keratinases (EC 3.4.99)[4]. 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[5].

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[6]. The prospective use of keratinases is in diverse applications where keratins should be hydrolyzed, such as the leather and detergent industries, textiles, waste bioconversion, medicine, and cosmetics for drug delivery through nails and degradation of keratinized skin[7]. 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[8]. The aim of present study was to investigate the presence of keratinase producing fungi in the indigenous soil where 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.

MATERIALS AND METHODS

Isolation and growth conditions

One hundred 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 grams) were collected from 25-30 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 was used[6]. Soil samples in duplicate were placed in sterilized Petri plates; moistened with 25% 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 29^0C. 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 surface of the Sabouraud's Dextrose Agar (SDA, Oxoid) and Czapek Dox Agar (CDA, Oxoid) and incubated at 30^0C. 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 60^0C as described by Guichard[9]. Dried feathers were ground into grinder (Anex Germany Products GMBh) till they converted into a fine powder.

Screening of fungi for keratinase production on skimmed milk agar

Skimmed Milk (Nido) was suspended in 10 ml distilled water. The ingredients listed in table 1 were dissolved in 90ml distilled water by heating when it was fully homogenized, then milk and homogenized agar were autoclaved separately at 12^0C for 15 minutes. After cooling to 45^0C, both were mixed and plated.

Table 1: Composition of skimmed milk agar.

Nutrients	Quantity (%)
$NaCl_2$ (Merck)	5%
Peptone (Oxoid)	3%
Agar (Oxoid)	2%
Skimmed Milk	1%
pH	7.0
Distilled water	100 ml

Submerged fermentation

The Submerged fermentation medium (Basal salt feather medium, table 2) was prepared as described by Hoq[10]. Conidia suspension of fungal spores containing 80,000 spores per ml (counted by Neubauer's chamber) was prepared in saline and incubated at 30^0C in an orbital shaking Incubator at 150rpm.

Crude Keratinase enzyme was collected by centrifugation of the culture at 6000rpm for 10 minutes at room temperature and filtered through an Acrodisc filter (0.2µm 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 a fine powder. The 5mg keratin azure powder was suspended in 1ml 50mmol/L Tris-HCl buffer (pH 8.0). The reaction mixture contained 1ml keratin azure suspension and 1ml crude enzyme. As a control, a 1ml keratin azure suspension and 1ml basal salt feather medium. As a control, a 1ml keratin azure suspension and 1ml basal salt feather medium. The reactions were carried out at 50°C in a water bath with constant agitation of 200r/min for 30 minutes. After incubation, the reactions were stopped by adding 2ml 0.4mol/L trichloroacetic acid (TCA) and followed by centrifuging at 3000×*g* for 5 minutes to remove the substrate. The supernatant was spectrophotometrically measured for release of the azo dye at 595nm 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 595nm under given conditions.

Table 2: Composition of basal salt feather medium.

Nutrients	Quantity (grams)
$NaCl_2$ (BioM Malaysia)	0.5
$MgCl_2$ (Sigma)	0.1
$CaCl_2$ (Sigma)	0.06
KH_2PO_4 (Merck)	0.7
Feather Meal (Prepared in this study)	10

Protein determination

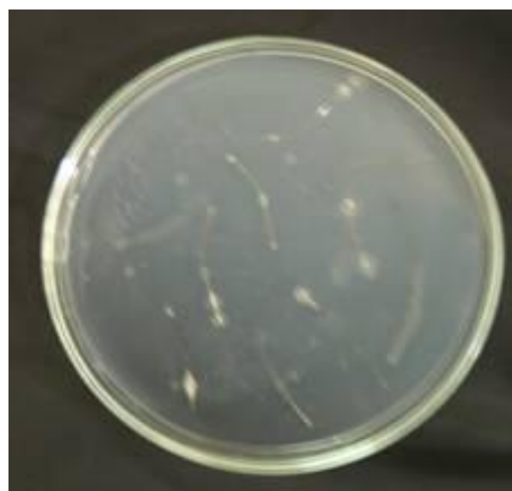
Protein concentration was measured by the method of Bradford[11], using bovine serum albumin (BSA) (Sigma, USA) as standard. The specific activity was expressed as the enzymatic activity (U) per mg of protein.

RESULTS

A total of 125 soil samples (25 from each sampling site) were collected for isolation of Keratinase producing fungi from the fertile lands, poultry farms, animal herds slaughter houses and barber shops from Khairpur district Sindh Pakistan.

The results of the isolation of Keratinase producing fungi are presented in table 3. The isolation was carried out using Hair Bait Technique and inoculating invaded hair on growth medium (Figure 1).

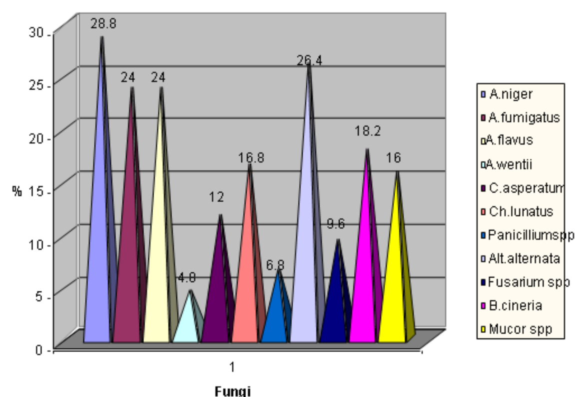
Figure 1: Invaded hair inoculation on Growth media.



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%), *Aspergillus flavus* (11.9%), *Aspergillus fumigatus* (11.1%), *Botrytis cinaria* (18.2%), *Chochliobolus lunatus* (16.8%), *Mucor spp* (16.0%), *Chrysporium asperatum* (9.9%), *Fusarium spp* (5.1%), *Penicillium spp*: (3.1%) and *Aspergillus 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 largest zone of hydrolysis at 30^{0}C.



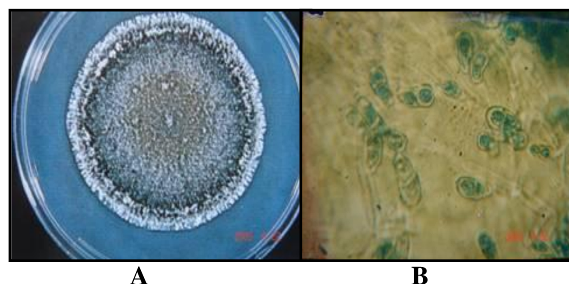
Effect of temperature

Effect of temperature ranging from 30^0 to 50^{0}C on the growth and zone of hydrolysis was determined on skimmed milk agar. The *C. lunatus* grown and produced 6mm and 2mm zones at 30, 35^{0}C and 40^{0}C respectively, where no zone was observed at 45^{0}C. The organism stopped growth at 50^{0}C.

Effect of carbon sources

The effect of different carbon sources on growth and enzyme activity of *C. lunatus* after one week of incubation at 30^{0}C. *C. lunatus* responded to carbon sources (Glucose, Maltose, Sucrose, Lactose and Cellulose) as assessed by zone on Skimmed Milk Agar. The zone of hydrolysis was observed in medium containing glucose (6mm zone size) maltose (5mm zone size) and sucrose (2mm zone size) but no zone was observed around growth in the medium containing lactose and cellulose.

Figure 2: Average percentage of fungal isolates.



A B

Figure 3: Isolation and identification of keratinolytic Cochliobolus lunatus by Hair Bait Technique (A) and Macro conidia (B) from Different Soil Samples in Khairpur.

Effect of nitrogen source

Effect of Nitrogen source (gelatin and peptone) was observed on the growth and proteolytic activity of *C. lunatus* at 30^{0}C and one weak incubation. Gelatin supported the growth and proteolytic activity of *C. lunatus* and produced the zone of hydrolysis (4mm) on Skimmed milk agar where as peptone did not support the growth and proteolytic activity.

Effect of metal ions

Effect of different metal ions on growth and proteolytic activity of Keratinase production from *C. lunatus* was observed using skimmed milk agar as a medium. NH_4Cl_2, KH_2 PO_4, K_2H PO_4, $NaCl_2$ supported the growth and $MgSO_4$ and $CaCl_2$ suppressed the growth of the *C. lunatus*.

Time course study for the production of keratinase from *C. lunatus*

Most of the commercial proteases are generally produced by submerged fermentation. *C. lunatus* was evaluated for its potential of producing keratinase enzyme by the submerged fermentation in basal salt feather medium at 30^{0}C. Substantial keratin-azure hydrolyzing activity was present in the culture fluid of keratin-containing media. *C. lunatus* produced 161 U/mg enzyme activities on 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[5]. The best studied are keratinases from the dermatophytic genera *Microsporum*[12]; and *Trichophyton*[13]. *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.[14].

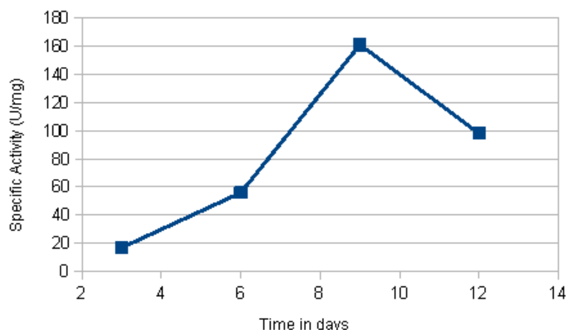


Figure 4: Time course study for production of keratinase from *C. lunatus.*

This study reports the production and characterization of keratinase from indigenous *C. lunatus.* The enzyme activity was revealed by the zone of hydrolysis on skimmed milk agar. The 30-40°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[15-17]. 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[18]. 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; showed 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 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[18-20] but Santos et al.[17] reported that the presence of glucose in the medium decreased the keratinolytic activity of *Aspergillus fumigatus* cultures that 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 Chopra and Mehta and Malviya et al.[21, 22] who reported maximum proteinase production with gelatin which served as poor substrate for fungal growth. The utilization of peptone was not favored may be 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 a metelloprotease.

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 keratinolytis 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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