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Biodegradation of Feathers by Keratinolytic Fungal Isolates from Poultry Farm Soil in Riyadh, Saudi Arabia

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The present investigation was aimed to isolate keratinolytic fungi from poultry farm soils of Riyadh and to study their ability to produce extracellular keratinase. Twenty three species from twelve genera were isolated by hair baiting technique. The most dominant in prevalence was *Aspergillus niger (13.1%)* followed by *Chrysosporium tropicum* (10.7%), *A.terreus* and *Fusarium oxysporum* (8.3%) each respectively. These isolates were screened for hydrolysis of keratin on solid keratin agar medium. Twelve fungal isolates showing strong keratinolytic activity were further used to study the synthesis of keratinase , during degradation of chicken feathers. All the isolates used, were able to degrade the keratin of chicken feathers producing total proteins and keratinase. The highest amount of protein was produced by *C.tropicum* (72.5µg/ml) followed by *F.oxysporum* (66.25µg/ml) and *A.nidulans* (63.65µg/ml), whereas *C.tropicum* (26.6KU/ml) produced the highest amount of keratinase followed by *A.nidulans* (19.8KU/ml) and the lowest amount by *Alternaria alternata* (8.3KU/ml) .There was a rise in the pH of the culture medium towards alkalinity. Hence our study not only highlights the abundance of keratinolytic fungi in poultry soils but also points at the potential of these fungi as excellent keratin degraders that can be used to solve the serious feather waste management problem, by converting them into usable products.

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Key words: Poultry soil, Keratinolytic fungi, Feathers, biodegradation, keratinase.

Today poultry industry is one the most important, gigantic and profitable sector in the business world .The poultry processing plant produces huge amounts of feather waste as by-products. Feather consist of a tough protein keratin, which is highly stable, durable and resistant to degradation; it owes this property to the presence of β keratin and the complex structural arrangement which is held by disulphide bridges and cross linkages[1]. Feather waste is a serious problem as environmental pollutant. Traditional ways of feather disposal like incineration not only needs large amounts of energy, but is also economically ineffective. While other physical or chemical methods of disposal into usable forms, like feather meal may result in loss of nutritionally essential amino acids such as methionine, lysine and tryptophan along with the formation of non-nutritive amino acids like lysinoalanine and lanthionine [2,3] . Hence, an important economical and ecofriendly alternative would be using the enzyme Keratinase.

Keratinolytic fungi are ubiquitous and soils which are rich in keratinous materials serve as their reservoir. They occur in nature mainly in the form of feathers, hairs, horns, hooves, nails and other cornified appendages and , constitute natural baits for these fungi [4]. These fungi produce special class of proteolytic enzymes called as “Keratinases” which degrade keratin by catalysing their hydrolysis. Keratinase facilitate dermatophytes to invade keratinized appendages of skin ,hair and nails causing mycosis. However,

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keratinolytic property has also been reported from various species of bacteria[5], actinomycetes[6] and fungi[7], besides dermatophytes[8]. Keratinase from fungi has various biotechnological and microbial applications. They are used in detergent, textile and leather industry and are utilized in the production of rare amino acids, like serine, cystiene and proline. Besides being used in conversion of feather meals for animal feed, they are also used in the making of biodegradable films, glues and nitrogen fertilizers for plants[9]. Our study was aimed to isolate and screen poultry farm soils of Riyadh, Saudi Arabia for the presence of keratinolytic fungi and further, determine the activity of extracellular keratinase in the degradation of feathers. To our knowledge this is the first report concerning, isolation of keratinolytic fungi from poultry farm soil samples from Riyadh.

MATERIALS AND METHODS

Isolation of Keratinolytic Fungi by Hair baiting technique

Poultry farms, on the outskirts of Riyadh were chosen for the study. 50gms of farm soil was collected from superficial layer, at a depth of 3-5 cms . Collection of samples was done in sterile polythene bags. Keratinolytic fungi were isolated using the Vanbreuseghem method[10]. Sterile Petri dishes were half filled with thoroughly homogenised soil samples and, the soil was moistened with sterile distilled water. It was then baited with short strands of sterilized defatted human hair .Strands of human hair were spread uniformly as baits on the surface of moistened soil samples. The plates were then incubated at 28±2° C and then examined daily from day 5 for any fungal growth over a period of 4 -8 weeks. Five replicates were set for each sample.

Samples of hair showing fungal growth were carefully observed, and then aseptically picked up and cultured on Sabourauds dextrose agar (SDA, Scharlau Chemie, Spain) supplemented with chloramphenicol (0.05mg/ml).

Identification

Identification of the purified fungal isolates was based on the monographs, using macro - and micro-morphological features [11-13]

Preparation of keratin substrate

Chicken feathers were defatted by soaking them in a mixture of chloroform-methanol (1:1, v/v) (Sigma Aldrich,USA) ,for 24 hours, washed finally with glass-distilled water and then dried in air. Pieces of feather about 1 cm length were weighed into portions of 500 mg.

Screening on keratin agar plates

All the fungi isolated from hair baits were further screened on agar plates following the method of Wawrzkiewicz *et al* [14] .Chicken feathers were used as source of keratin. Feathers were dissolved in dimethyl sulphoxide (Sigma Aldrich,USA) and precipitated with acetone. This precipitate was added to sterile agar medium, as a sole source of Carbon and Nitrogen. Keratinolytic activity of the fungi was detected as a clear zone around the colony after incubation for 8 days at room temperature. Diameter of the clear zone was measured to quantify keratinolytic activity.

Preparation of mineral medium and spore suspension.

Mineral medium was used for assay for keratin degradation with slight modification (Wawrzkiewicz *et al* [14]. Di-Potassium hydrogen O-phosphate-1.500g; Magnesium sulfate-0.050g; Calcium chloride-0.025g; Ferrous sulfate-0.015g; Zinc sulfate-0.005g. (Sigma Aldrich,USA) ; Distilled water-1000ml; Sterile defatted feather -500mg; pH-7.5. Spore suspension of the fungal isolates was prepared by adding a 10 ml of sterilized distil water to 10 days old culture .1 ml of this suspension, containing 10^6 - 10^7 spores, was added as an inoculum to each flask, containing mineral medium[15].

Submerged cultivation

50 ml of the prepared mineral medium with 500 mg defatted feathers was distributed in 250 ml Erlenmayer's flasks and was sterilized at 121°C for 15 minutes. It was then inoculated aseptically with 1 ml of spore suspension, incubated at 28±1°C in a shaker (120 rpm) for a period of 15 days .During the period , samples were checked daily and the feather was considered to be degraded when the pieces of 0.5mm -0.2mm or smaller remained. For each fungal species, four test flasks and one control set were maintained .Flasks containing the medium without inocula served as control. After complete degradation culture filtrate was collected, centrifuged at 2000 rpm for 30 minutes, to remove the spores. The supernatant was filtered through Whatmans filter paper No 1 . The filtrate so obtained

was further used to study the biochemical changes associated with complete biodegradation of feathers such as pH, protein released and keratinolytic activity.

Determination of pH

The pH of the culture filtrate was determined using pH meter. (Systronics pH meter).

Estimation of protein

The protein content in the culture filtrate was determined by the Bradford method[16]. Bovine serum Albumin.(Sigma Aldrich,USA) was used as standard and the absorbance was read at 595mm.

Determination of Keratinolytic Activity

Keratinolytic activity was assayed by a modified method of Yu *et al*., [17] and chicken feathers were used as substrate. 20 mg of poultry feathers were cut into 1-3 mm long bits and were suspended in 3.5ml of Tris-HCl buffer (0.1M, pH7.8) to which 0.2 ml of culture filtrate (enzyme source) was added. The mixture was kept in a water bath at 37^{o}C for 1 h, after incubation, the assay mixture was dipped in ice cold water for 10 min and the remaining feather were filtered out. The optical density of clear mixture was measured by UV-Visible- Spectrophotometer at 280nm (Schimadzu,USA) against corresponding blank that was prepared in the same way, instead of enzyme solution, buffer was added.

Enzyme Unit

One unit of Keratinolytic activity (KU) was the amount of enzyme that could liberate products having an absorbance of 0.1 under the assay condition[18] (1KU=0.100 corrected absorbance)

RESULTS AND DISCUSSION

Isolation of keratinolytic fungi

Twenty three species belonging to twelve genera were isolated from poultry farm soil by hair baiting technique. The most dominant in prevalence was *Aspergillus niger* (13.1%), followed Table 1 by *Chrysosporium tropicum (10.7%*), *A.terreus* and *Fusarium oxysporum* (8.3%) each respectively. The genus *Aspergillus* dominated with five species followed by *Chrysosporium* representing 4 species. In a study conducted on poultry farm soil, a total of 106 filamentous fungi were isolated these included thirteen species belonging to four genera viz.*Aspergillus, Alternaria, Curvularia* and *Penicillium*[19]. Ingle *et al*., [20] isolated 14 genera and many non sporulating forms of fungi from poultry farm soils of Nanded, Maharastra and all of them possessed variable levels of keratinolytic property. Our findings clearly indicate the richness of the poultry farm soils with varied non- dermatophytic fungi as they were isolated from all the sites screened and most have shown considerable keratin degradation capability on hair baits. This wide array of fungal isolation could be due to the presence of heavy loads of keratin waste which is added to soil, from the poultry birds especially in the form of feathers. In natural environments these fungi are involved in recycling of carbon , nitrogen and sulphur of the keratins[21]. Hence, the presence and distribution of these fungi, largely seem to depend on keratin availability.

Screening for keratinolysis on solid medium

All the fungi isolated from hair baits were further screened on keratin agar plates. Strong keratinolytic activity ,with a clear zone between 21-51 mm was shown by 52.2% of the isolates, where as 30.4% of the fungi possessed moderate to weak activity (below 21mm) and 17.4% of the fungal isolates did not clarify the keratin agar .The maximum diameter of clear zone was shown by *C. tropicum* (62mm), followed by *Fusarium* sp (50 mm), *F.oxysporum* (44 mm) and *A. nidulans* (42mm). All readings were taken on 8th day. Several researchers have screened the ability of fungi to degrade keratin in solid agar and similar mediums[3,22] . Fredrich *et al*., [15] screened 300 fungal strains on agar plates containing soluble keratin .They observed that 54% of these fungi were able to hydrolyse the protein by forming a clear zone around the colonies.

Submerged culture: Changes in pH.

Twelve most active fungal isolates on agar plates were chosen, to test their ability to degrade feathers in submerged shaken cultures. All the strains caused an increase in pH of the culture medium during keratin degradation, ranging between 7.8 to 9.5. As the keratinolysis progressed the pH of the medium increased, it was also observed that complete degradation caused by strong keratinolytic fungi, rendered the medium more alkaline than those which were less keratinolytic. Hence pH of the medium is a significant factor that influences the physiology of a microorganism. It has been stated that a pH of

7 to 9 supports keratinase production and feather degradation in most microorganisms [23]. This increase in pH is due to the ability of keratinolytic fungi to extract nutrients from substrates and thereby cause the release of cysteine, keratinase and proteins[24,25]. Additionally, when saprophytic fungi are grown on keratin substrate, rise in pH level of the culture medium, is attributed to the accumulation of ammonium ions during deamination, as suggested by several workers[17,26,27].

Protein and keratinase released during keratin degradation All the fungal isolates used in this study were able to degrade feathers in the culture medium and consequently release the protein at the end of experimental period, though the degradation period and amount of protein released varied with different fungal isolates. It was observed that *C.tropicum* (72.5µg/ml) was able to release the highest amount of protein followed by *F.oxysporum*, (66.2µg/ml),*A. nidulans*(63.6µg/ml) and *C.indicum* (56.4µg /ml) while the lowest was by *C. keratinophilum* (26.8µg/ml) and *Alternaria alternata* (29.7µg/ml) respectively. Our results are in accordance with several other workers who have reported that keratinolysis is caused by both dermatophytes and non dermatophytic fungi and have resulted in the release variable amounts of protein in both stationary and static cultures [28,29]. Similarly Kim [30] showed, all the five *Aspergillus* species used in his study were able to release different amounts of total proteins in the medium

Highest amount of keratinase was released by *C.tropicum (26.6 KU/ml)* and lowest by *A. alternata (8.3 KU/ml)*. Both *A. niger* and *A. nidulans* caused complete degradation in 5 days ,while *Chrysopsorium* spp and other fungi took 7-10 days . Elaides *et al*.,[31] selected 32 non dermatophytic fungi to test their ability to produce keratinase in alkaline submerged shaken cultures. They reported *A. niger, C.cladosporioides, Metarrhizium anisopliae, Neurospora tetrasperma* were the best producers with levels higher than 1.2 U ml^{-1} keratinase at alkaline pH. Yet in another study *Chrysoporium sp* and *Microsporum sp* under similar conditions caused feather decomposition releasing variable amounts of keratinase[32]. Several Researchers have shown Keratinases production by fungi, such as *C.tropicum*[33]; *Alternaria, Chrysopsorium, Paecilomyces, Penicillium,Curvularia* and several *Aspergillus* sp [34, 35].

Gupta and Ramnani[9], stated that keratinolytic activity of microorganisms is normally associated with the production of keratinase,serine protease or metalloproteases, irrespective of the microorganisms. The other explanation for variable amounts of both protein and enzyme found in test

Table 1. Percentage occurrence of keratinolytic fungi in poultry farm soils on hair baits and keratinolytic activity on agar plates

Fungi isolated	% occurrence	clear zone diameter (mm)
Acremonium sp	2.4%	12
Alternaria alternata	4.8%	34
Alternaria sp	1.2%	18
Aspergillus flavus	7.1%	37
A.fumigatus	6.0%	28
A.nidulans	4.8%	42
A.niger	13.1%	36
A.terreus	8.3%	25
Aspergillus sp	1.2%	0
Chaetomium globossum	1.2%	0
Chrysosporium indicum	7.1%	38
C.tropicum	10.7%	62
C.keratinophillum	2.4%	28
Chrysopsorium.sp	1.2%	35
Curvularia lunata	2.4%	6
Fusarium oxysporum	8.3%	44
Fusarium sp	2.4%	50
Mucor sp	3.6%	0
Penicillium chrysogenum	4.8%	2
Penicillium sp	1.2%	16
Paecilomyces sp	2.4%	4
Trichoderma sp	1.2%	12
Verticillium sp	2.4%	0
	100.0%	

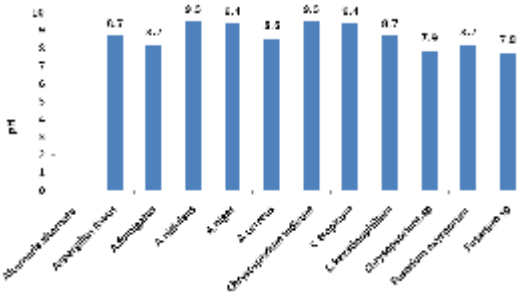

Fig. 1. Changes in the pH of the medium due to keratinolysis by fungal isolates.

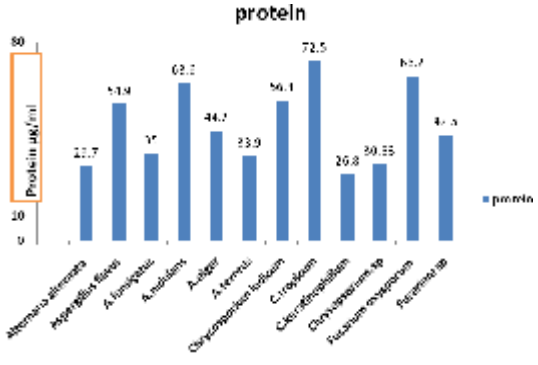

Fig. 2. Release of protein(µg/ml) during keratinolysis by different fungal isolates

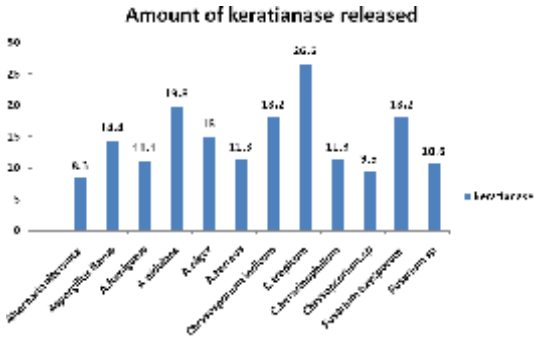

Fig. 3. Amount of Keratinase KU/ml released during keratinolysis by different fungal isolates

medium could be the presence and interaction of products during keratinolysis. It was stated that the lower levels of degradation, may be due to restricted substrate specificity of the enzyme or removal of some accessory proteins capable of splitting the disulphide bonds present in the keratin molecules during the hydrolysis process[36]. Therefore the strength of keratinolytic activity depends on the ability of these fungi to release soluble sulphur-containing amino acids and polypeptides into medium[37]. These amino acids may enter the protein pool of the fungus, thereby enhancing its growth and simultaneous keratinase production[24].

Hence, the process of keratinolysis is complex and has not been understood thoroughly. The protein keratin offers great resistance to its degradation due to high mechanical stability conferred by the cysteine interelinkage making it a compact molecule and, difficult to break down. Therefore, capability of filamentous fungi to degrade keratin may be the result of a combination of extracellular keratinase, mechanical keratinolysis (mycelial pressure and/or penetration of the keratinous substrate), sulphitolysis (reduction of disulphide bonds by sulphite excreted by mycelia) and proteolysis[9]. Enzymatic or chemical reducing agents in the form of disulfide reductases, sulfite, thiosulfate or cellular membrane potential may play a significant role in the degradation of this insoluble protein, additionally, the initial attack by keratinases and disulfide reductases may allow other less specific proteases to act, resulting in an extensive keratin hydrolysis [27].

CONCLUSION

We can conclude that Poultry farm soils are rich reservoirs of keratinolytic fungi. These fungi can be grown on media with feathers as its sole source of carbon and nitrogen, and degrade them releasing reasonable amount of enzyme and protein .Hence, the tremendous amount of poultry feather waste which is added as an environmental pollutant, can be commercially exploited as an inexpensive substrate resulting in its removal at a very low cost.

Additionally, it can be converted into nutritional feed additives. Therefore, further studies to evaluate the biotechnological potential of these fungi ,to convert them into more useful hydrolysis products are needed. Our study not only highlights the abundance of keratinolytic fungi in poultry soils but also points at the potential of these fungi as excellent keratin degraders that can be used in biotechnology industry for various purposes .

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