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Degradation of Keratins by *Chrysosporium* species: A Comparative Study

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Keratin degrading ability of three keratinophilic fungi was studied *in vitro* in presence of different keratinous substrates viz hairs, feathers and human nails. *Chrysosporium tropicum* (NFCCI-3317), *C. keratinophilum* (NFCCI-3318) and *C. pannicola* (NFCCI-3362) were tested to assess their biodegradation capability and keratin invasion for a period of six weeks. *C. tropicum* colonize feathers as early as 4 days, hair by 6 days and human nails by 17 days. *C. keratinophilum* and *C. pannicola* colonize feathers on 5th and 6th days respectively whereas both colonize hair and nails respectively on 7th and 18th. Complete feathers and hairs degradation was observed by the three *Chrysosporium* species within 4 weeks but degradation of nails was only 40% after 6 weeks of observation. *C. tropicum* showed the highest enzyme activity and *C. keratinophilum* the least.

Key words: Keratin, Keratinophilic fungi, Biodegradation, *Chrysosporium*.

Keratin is an insoluble filamentous protein found in mammalian tissues like skin, hair, nail, horn, hoofs and also in birds' feathers. Hardness of keratin depends on the composition of cystine and cysteine. Hard keratins are rich in cystine like hair, feather, and nail, horn, etc. whereas soft keratin like epithelia and silk lacks both cystine and cysteine and rich in other amino acids such as glycine, alanine and serine[1]. Biodegradation of hard keratin is more important as accumulation of it has far reaching impact on ecological imbalances. Despite being hard, keratin can be efficiently degraded by groups of different types of microorganisms. Fungi are found to be one of such group which has the potential to invade keratins and degrade it mechanically and enzymatically[2]. Mechanical invasion of hair by fungi was demonstrated by various workers[3-6]. Production of fungal extracellular keratinase enzymes and its keratinolytic potential was studied by different workers[7–13]. Three different *Chrysosporium* species viz. *C. tropicum*, *C. keratinophilum* and *C. pannicola* were isolated from the soil of this area[14] and in the present study their ability to degrade various keratinous substrates were studied.

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MATERIALS AND METHODS

Keratin invasion test

The test was performed following the protocol of Salkin et al.[15] with minor modifications. Hairs, feathers and nails were cut into short segments (approx. 1 cm), defatted and autoclaved at 120°C and 15 lbs for 15 min. After cooling 10 to 15 segments of hairs, feathers and nails were separately placed in sterile 90mm Petri plates. 20 ml of sterile de-ionized distilled water and a few drops of 10% yeast extract solution were aseptically added into each plate. A small portion of growth from 7 to 10 days old culture of *C. tropicum*, *C.*

keratinophilum and *C. pannicola* were placed into each plate and incubated at 26±1°C for 6 weeks. The tests were carried out in triplicates for each substrate and for each fugal culture. Appearance of fungal growth on keratin segments were observed periodically. Keratin segment with associated fungal mycelia was removed after every week, mounted in lactophenol cotton blue and examined microscopically to observe fungal invasion, if any, in keratinous substrates.

For production of extracellular enzyme keratinase and to assess the keratinolysis basal salts medium [KH_2PO_4 - 1.5g; $MgSO_4$, $7H_2O$ - 0.025g; $FeSO_4$, $7H_2O$ - 0.015g; $ZnSO_4$, $7H_2O$ - 0.005g and $CaCl_2$ - 0.025g in 1 lt. deionized distilled water, pH – 7] was used for each fungal species. Erlenmeyer flasks (150 ml) containing 50 ml of sterilized basal salts medium supplemented with 500 mg of defatted and pre-sterilized human hairs of 1 cm length as a keratin source were inoculated and incubated at 27°C for 3 weeks in static condition. In test sample, a 6 mm disc from 7 days old fungal culture was used as inoculums. Flasks containing the medium with a disc of agar without the fungus served as control. For each species, six test flask and one control set were maintained for observations.

After the incubation period culture filtrates from each flask were filtered through Whatman filter paper 42 and centrifuged at 5000 rpm for 5 min. The supernatant was used for estimation of extracellular keratinase enzyme and protein.

Keratinase enzyme assay

Keratinase activity was assayed following the method of Yu et al.[7] with modifications. The substrate was human hair extracted with chloroform-methanol (1:1), air dried and sterilized. 50 mg of hair (4-5 mm) was suspended in 4.5 ml of 0.028M phosphate buffer to which 0.5 ml of culture filtrate was added as enzyme source. The reaction mixture was incubated at 37°C for 1 hr. and then immersed in ice water for 10 min to stop the reaction. The remaining hair was removed by filtration through Whatman filter paper No. 42 and the absorbance of the filtrate was measured at 280 nm (UV-VIS Spectrophotometer, SHIMADZU). One unit of the keratinase activity was defined as the amount of enzyme that increases absorbance by 0.1 under the conditions described.

RESULTS AND DISCUSSION

Appearance of fungal mycelia on feathers was first observed on 4th day in case of *C. tropicum* followed by *C. keratinophilum* and *C. pannicola* in 5 and 6 days respectively (Table 1). On hair segment, inoculated with these species, colonization first observed on 6th day by *C. keratinophilum* and *C. tropicum*. For *C. pannicola* mycelia growth observed on 7th day (Table 1). Whereas on nails colonization started late and on 17th day it was observed for *C. tropicum* and on the very next day for *C. keratinophilum* and *C. pannicola* (Table 1). All the three species showed complete degradation of feathers and hairs after 4 weeks of incubation. More than 80% degradation was complete by the end of third week of observation, so day wise complete digestion of feathers and hairs was achieved around 25th to 26th day. The situation was quite different in case of nail; the fungal species were unable to completely digest it within 6 weeks of incubation. Around 35 to 40% degradation was achieved and *C. keratinophilum* showed best result in our study followed by *C. tropicum* and *C. pannicola* respectively (Table 1).

The highest amount of enzyme was produced by *C. tropicum* (8.82 KU/ml) followed by *C. pannicola* (6.01 KU/ml) and *C. keratinophilum* (5.85 KU/ml) in basal salts media where human hairs was used as source of keratin substrate. Though production of enzyme was highest for *C. tropicum* but other species showed good keratinolytic activity which is evident from the increase in pH (Table 2) of media after 3 weeks of incubation.

C. keratinophilum produces perforating organs (Fig. 1) which forms deep troughs in feather and hair and growing mycelium forces its way longitudinally through cortex and medulla. *C. tropicum* similarly produces perforating organs on hair and feather. English[16] proposed that a perforating organ is a modified mycelium; younger one develops clearly visible cone-shaped projection on hair surface (Fig. 2). The older organ develops quite sharp pointed edge (Fig. 1) and also gives rise to lateral perforating organ which runs through the cortex of the hair or feathers (Fig. 3 & 6). Lateral mycelia growth through cortex, the hardest part of hair and feather, as our observation

Table 1. Percent invasion of human hair by different *Chrysosporium* species

Species	Colonization (days)	Percent invasion of individual hair segment at weekly interval (Mean ± SE)					
		1	2	3	4	5	6
CK	7	40.33 ± 1.19	64.33 ± 0.72	81.67 ± 1.65	100.00 ± 0.00	-	-
CP	7	41.67 ± 0.72	66.67 ± 1.44	86.67 ± 0.98	100.00 ± 0.00	-	-
CT	6	43.67 ± 1.19	70.67 ± 1.44	87.33 ± 0.54	100.00 ± 0.00	-	-
Percent invasion of individual feather segment at weekly interval (Mean ± SE)							
CK	5	40.33 ± 1.91	65.33 ± 4.25	88.67 ± 0.72	100.00 ± 0.00	-	-
CP	6	44.67 ± 1.66	62.00 ± 2.16	91.33 ± 3.81	100.00 ± 0.00	-	-
CT	4	42.00 ± 2.05	67.00 ± 1.89	88.33 ± 1.91	100.00 ± 0.00	-	-
Percent invasion of individual nail segment at weekly interval (Mean ± SE)							
CK	18	0.00 ± 0.00	0.00 ± 0.00	4.67 ± 0.72	12.67 ± 0.73	24.33 ± 0.98	40.33 ± 1.52
CP	18	0.00 ± 0.00	0.00 ± 0.00	5.00 ± 0.47	11.67 ± 0.98	23.33 ± 1.19	35.33 ± 1.19
CT	17	0.00 ± 0.00	0.00 ± 0.00	7.33 ± 0.98	14.00 ± 1.25	21.00 ± 2.05	38.67 ± 1.51

CK – *C. keratinophilum*; CT – *C. tropicum*; CP – *C. pannicola*

suggest the possible mechanism of disintegration of keratin fibers from inside (Fig. 6 & 7) and made it more vulnerable to enzymatic digestion by the keratinase enzyme produced by the organism[2].

Katiyar and Kushwaha[4] demonstrated complete digestion hair after 24 days by *C. tropicum*. Sabino et al.[5] demonstrated that *C. keratinophilum* had strong keratinophilic activity with 100% hair degradation capability. Similarly *C. pannicola*, an active agent of keratinolysis, showed hair invasion and complete digestion[17].

All the three fungi demonstrated surface erosion, uniform and localized, as one of the means of digestion of keratins (Table 1), also demonstrated by Mitola et al.[18]. Surface erosion means progressive uniform digestion on the whole surface or on localized areas of keratin[19]. In our study *C. pannicola* showed more prominence towards surface erosion (Fig. 4) not perforation, contrary to the observation of Kushwaha[17]. Likewise, Marchisio[19] observed only surface erosion of *C. tropicum*, which is contrary to our present findings. In nail, we observed surface erosion mode of invasion and no perforating organs, also demonstrated by English[16], probably because of the hardness of nail.

Singh[20] demonstrated that *C. keratinophilum* produce 21.0 and 18.0 KU/ml of enzyme in a different set of experimental media with respectively buffalo tail hair and buffalo skin as a substrate. He also demonstrated percent keratin degradation was 44.2 and 53.0 respectively for buffalo tail hair and buffalo skin in 15 days. Faterpekar et al.[21] demonstrated 59 and 52% degradation of hair by *C. keratinophilum* and *C. tropicum* respectively in basal salts media after 6 weeks of incubation. *C. keratinophilum* degraded 81% poultry feathers in basal salts medium in 30 days and at same condition *C. pannicola* and *C. tropicum* were able to digest 31 and 61% respectively[22]. Maruthi et al.[23] observed maximum production of enzyme by *C. tropicum* was 8.56 KU/ml with hair as a substrate and increase in pH was 9.2.

Keratinase is an inducible enzyme[2] and it release is not the sole indicator of keratinolysis. It is the combined effort of mechanical and enzymatic action of the fungi which can be gauged from the hair or feather perforation test and percent invasion, production of enzyme, its release of protein and

Plate depicting different stages of invasion on feather, hair and nail by *Chrysosporium* species

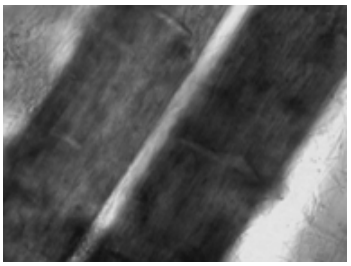

Fig. 1. Perforating hyphae of *C. keratinophilum* on feather and hair after 1st week (x400)

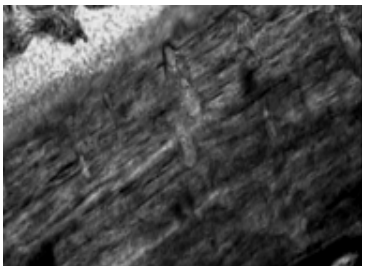

Fig.2. Perforating hyphae of *C. tropicum* on hair after 2nd week (x400)

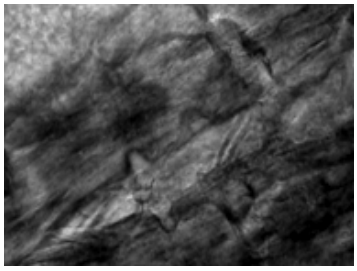

Fig. 3. Perforating hyphae along with medullary perforator of *C. tropicum* (x1000; Oil)

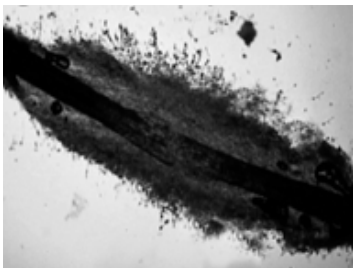

Fig. 4. Surface erosion and transverse breakdown by *C. pannicola* after 2nd week (x100)

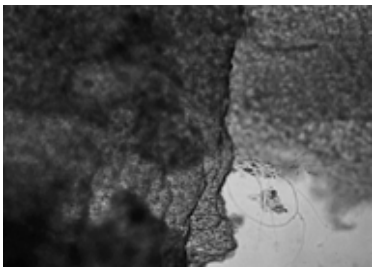

Fig. 5. Surface erosion of nail by *C. tropicum* after 4th week (x400)

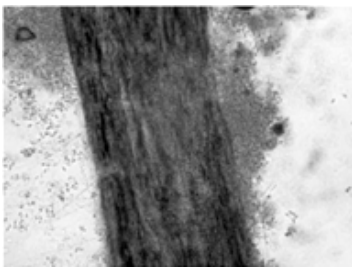

Fig. 6. Mycelial growth through medulla and Cortex after 3rd week (x200)

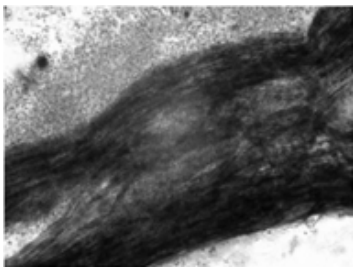

Fig. 7. Cuticle lifting of hair by *C. keratinophilum* (x200)

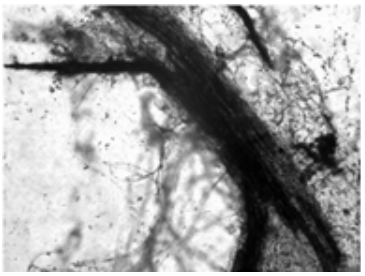

Fig. 8. 80%Digestion of hair by *C. keratinophilum* after 3rd week (x100)

Table 2. Change in pH and enzyme activity by different *Chrysosporium* species

Species	Changes in pHMean ± SE	Enzyme (Units/ml) Mean ± SE	Modes of invasion
CK	8.77 ± 0.10	5.65 ± 0.07	Perforating hyphae; surface erosion; cuticle lifting and disruption
CP	8.64 ± 0.09	6.01± 0.19	Uniform and localized surface erosion; transverse breakdown
CT	8.86 ± 0.08	8.82 ± 0.36	Perforating hyphae; medullary perforator; surface erosion

increase in pH of the media. pH, a good indicator of keratinolysis, suggest digestion of keratin protein, deamination and production of ammonia[24].

CONCLUSION

In our study all three *Chrysosporium* species showed 100% degradation of hair and feather within 4 weeks of incubation. However, degradation of nail was slow on account of its hardness as compared to other keratin substrates. Good production of enzyme and increase in pH suggest efficient keratinolytic ability of these fungi. But no correlation was found between keratinase production and degradation of keratin substrates as mechanical capability of these fungi were also involved in degradation. So, degradation of keratins was achieved by the coherent actions of keratinase and the mechanical activity of perforating organs of these fungi. Thus they are of great use as agent for biodegradation of hard keratin and the enzyme could be of huge industrial applications.

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REFERENCES

1. Moll, R., Divo, M., Langbein, L. The human keratins: biology and pathology. *Histochem Cell Biol*., 2008; **129**: 705-733.
2. Marchisio, V.F. Keratinophilic fungi: their role in nature and degradation of keratinic substrate. In: *Biology of Dermatophytes and other Keratinophilic Fungi* (Kushwaha RKS, Guarro J, eds). Spain: Revista Iberoamericana de Micologia, 2000; pp 86-92.
3. Vanbreuseghem, R. Keratin digestion by dermatophytes: a specific diagnostic method. *Mycologia*, 1952; **44**: 176-182.
4. Katiyar, S., Kushwaha, R.K.S. Invasion and biodegradation of hair by house dust fungi. *Int. Biodeterior. Biodegrad*., 2002; **50**: 89-93.
5. Sabino, R., Verissimo, C., Brandao, J., Alves, C., Alves, P., Parada, H., Rosado, L. Keratinophilic fungi: a microscopic approach by in vitro hair perforation analysis. *Microsc. Microanal*., 2008; **14**: 115-118.
6. Blyskal, B. Fungi utilizing keratinous substrate. *Int. Biodeterior. Biodegrad*., 2009; **63**: 631-653.
7. Yu, R.J., Harmon, S.R., Blank, F. Isolation and purification of extracellular keratinase of *Tricophyton mentagrophytes*. *J Bact*., 1968; **96**: 1435-1436.
8. Malviya, H.K., Rajak, R.C., Hasija, S.K. Purification and characterization of two extracellular keratinases of *Scopulariopsis brevicaulis*. *Mycopathologia*, 1992; **119**: 161-165.
9. Gradiser, H., Kern, S., Friedrich, J. Keratinases of *Doratomyces microsporus*. *App. Microbiol. Biotechnol*. 2000; **53**: 196-200.
10. Bernal, C., Vidal, L., Valdivieso, E., Coello, N. Keratinolytic activity of *Kocuria rosea*. *World J. Microb. Biot*., 2003; **19**: 255-261.
11. Gradiser, H., Friedrich, J., Krizaj, I., Jerala, R. Similarities and specificities of fungal keratinolytic proteases: comparison of keratinases of *Paecilomyces marquandii* and *Doratomyces microsporus* to some known proteases. *App. Env. Microbiol*., 2005; **71**: 3420-3426.
12. Bernal, C., Cairo, J., Coello, N. Purification and characterization of a novel exocellular keratinase from *Kocuria rosea*. *Enz. Microbial. Technol*., 2006; **38**: 49-54.
13. Anbu, P., Hilda, A., Sur, H.W., Hur, B.K., Jayanthi, S. Extracellular keratinase from *Trichophyton* sp. HA-2 isolated from feather dumping soil. *Int. Biodeterior. Biodegrad*., 2008; **62**: 287-292.

14. Sarkar, A.K., Rai, V., Gupta, A.K. Incidence of keratinophilic fungi in areas of raipur city, Chhattisgarh, India. *Afr. J. Microbiol. Res.*, 2014; **8**: 264-269.
15. Salkin, I.F., Hollick, G.E., Hurd, N.J. Kemna, M.E. Evaluation of human hair sources for the *in vitro* hair perforation test. *J. Clin. Microbiol*., 1985; **22**: 1048-1049.
16. English, M.P. The saprophytic growth of keratinophilic fungi on keratin. *Sabouraudia*, 1963; **2**: 115-130.
17. Kushwaha, R.K.S. The genus *Chrysosporium* its physiology and biotechnological potential. In: *Biology of Dermatophytes and other Keratinophilic Fungi* (Kushwaha RKS, Guarro J eds). Spain: Revista Iberoamericana de Micologia, 2000; pp. 66-76.
18. Mitola, G., Escalona, F., Salas, R., Garcia, E., Ledesma, A. Morphological characterization of in vitro human hair keratinolysis, produced by identified wild strains of *Chrysosporium* species. *Mycopathologia*, 2002; **156**: 163-169.
19. Marchisio, V.F., Fusconi, A., Rigo, S. Keratinolysis and its morphological expression in hair digestion by airborne fungi. *Mycopathologia*, 1994; **127**: 103-115.
20. Singh, C.J. Optimization of an extracellular protease of *Chrysosporium keratinophilum* and its potential in bioremediation of keratinic wastes. *Mycopathologia*, 2002; **156**: 151-156.
21. Faterpekar, S.K., Jain, S.K., Shrivastav, A. Degradation of horse hair by soil inhabiting keratinophilic fungi. *J Cell Tissue Res* 2008; **8**: 1471-1476.
22. Parihar, P., Kushwaha, R.K.S. A survey of keratinophilic fungi as a tool for hen feather utilization. *Mycoscience*, 2000; **41**: 645-649.
23. Maruthi, A.Y., Lakshmi, A.K., Rao, R.S., Chaitanya, A.D. Degradation of feather and hair by *Chrysosporium tropicum*: a potent keratinophilic fungus. *Afr. J. Biotechnol*., 2011; **10**: 3579-3584.
24. Kunert, J. Physiology of keratinophilic fungi. In: *Biology of Dermatophytes and other Keratinophilic Fungi* (Kushwaha RKS, Guarro J eds). Spain: Revista Iberoamericana de Micologia, 2000; pp. 77-85.