GENERAL SUMMARY
Soil inhabiting keratinophilic fungi are natural colonizers of keratinic substances and are playing an important role in their degradation process. Keratin are sclero-proteins composed of long polypeptide chains having high sulphur content due to the presence of sulphur containing amino acids i.e., cysteine, cystine and methionine. The disulphide bonds are considered to be responsible for the stability of keratin and its resistance to most proteases. Keratinophilic fungi have a versatile range for their nutrition and are adapted to the utilization of proteins as the main source of nutrition. They produce extracellular keratinase and other enzymes including proteases for complete hydrolysis of natural keratins. Keratin degradation accomplished by three major processes namely deamination, sulphitolyis and proteolysis, which results in release of proteins, peptides and mixture of free amino acids which can be used for animal feed upgradation.

In the present investigation an attempt has been made to isolate keratinophilic and other keratin loving fungi from soils of different places i.e., District, Damoh and Sagar of Madhya Pradesh and Bilaspur and Korba of Chhatisgarh. The samples collected from poultry farm, cattle farm, municipal gardens, road sides and agriculture fields of surveyed areas were baited for the isolation of keratinophilic fungi and other keratin degrading moulds. Keratinic substances like human hair, nails, hen feathers and natural wool were used as baits for the isolation purposes.
Occurrence of keratinophilic and related keratin loving fungi was noted only in 61.11, 58.97, 51.85, 50.0 and 45.45 percent samples of cattle farm, poultry farm, municipal garden, road sides and agriculture fields, respectively. A total of 171 fungi belonging to 43 species of 14 different genera were isolated from 150 soil samples collected from different habitats. Maximum number of fungal species were isolated using hen feathers as keratin baits while only 21, 15 and 13 fungal species were isolated using human hair, human nails and natural wool, respectively.

In nature the keratin is found as appendages of the skin of man and animals formed as a consequence of keratinization of tissues. The natural keratins are not the purified form of keratin proteins but found associated with certain other substances mainly the secretions of sebaceous glands of mammals and preening glands in birds. And hence, for the complete degradation of natural keratins not only keratinases but other enzymes are also required.

In bioprocessing of materials fungal enzymes are gaining importance in general, while keratinases, proteases and lipases are specifically significant in the bioconversion of keratinic waste into value added products. In the present study, a total of 58 fungal strains have been tested for the production of lipase, protease and keratin degrading capabilities. Lipase activity of test fungi was assayed using the method of Hankins and Anagnostakis (1975). Sorbitan monolaurate was used as lipid substrate. All the test fungi have been found positive for lipase activity. Test strains of Chrysosporium indicum (CH1, CH2, CH4),
*Chrysosporium pannicola* (CH10), *Chrysosporium* sp. (CH7, CH15, CH19), *Malbranchea aurantica* (MA1), *Keratinophyton terrium* (K1 and K2), *Verticillium* sp. (V1) have shown greater activity of lipase than other test organisms. In all these cases relative enzyme activity (ratio of zone of enzyme activity around colony and the diameter of fungal colony) was found more than two. The protease activity was determined using the same method as described for lipase activity but the substrate used was gelatin (0.4%). Only 53 test strains have shown protease activity around their growing colonies on gelatin containing agar medium. Ten test strains including four of *Chrysosporium indicum* (CH1, CH2, CH3, CH4) and one each of *Chrysosporium pannicola* (CH10), *Chrysosporium* sp. (CH5, CH7, CH15, CH17) and *Verticillium* sp. (V1) have shown relatively greater activity of protease than other protease positive strains.

To determine the keratin degrading capability of these fungi they were grown in mineral salt solution containing natural human hair and hen feathers as sole source of nutrients. Loss in weight of hair and feathers was determined after 20 days of incubation at 28°C temperature. All the test fungal strains have caused a loss in weight of hair and feathers during their growth on human hair and hen feathers. Except in few cases the test strains degraded greater amount of hen feather than human hair. *Malbranchea* sp. (MA3), *Fusarium oxysporum* (F7), *Chrysosporium indicum* (CH1, CH3), *Microsporum gypseum* (MI3) and *Fusarium chlamydosporum* (F8) degraded 57.50, 58.40, 44.45, 42.60, 41.75 and 41.65 per cent hen feathers, respectively in 20 days.
The presence of oil and fats in keratinic substances of man and animal origin is natural. Whether presence of such substances is of any significance in the process of their colonization is a subject of enquiry. In order to know the nature and capabilities of some selected keratinophilic and other keratin loving fungi they were further studied for the production of extracellular lipase, keratinase and other proteases (gelatinase and caseinase) utilizing natural and defatted human hair and hen feathers as sole source of nutrients and the substrate. For this the test fungal strains i.e., four of *Chrysosporium indicum* (CH1, CH2, CH3, CH4), two of *Microsporum gypseum* (MI2, MI3) and one strain of *Chrysosporium keratinophilum* (CH6), *Chrysosporium pannicola* (CH10), *Chrysosporium* sp. (CH7), *Fusarium chlamydosporum* (F8), *Fusarium oxysporum* (F7), *Fusarium* sp. (F13), *Malbranchea* sp. (MA3), *Trichophyton* sp. (T1) and *Verticillium* sp. (V1) were grown in mineral salt solution containing natural and defatted human hair and hen feathers. The cultures were grown for 20 days and examined to determine the total weight loss in test keratin substrates. Release of protein and amino acids was also quantified to confirm keratinolytic nature of test organism in each case. *Fusarium oxysporum* (F7) caused 51.50±0.50 and 47.00±1.0 percent loss in weight of defatted and natural feathers, respectively. On the basis of data on *in vitro* degradation of natural and defatted keratinic substrate it can be concluded that the presence of oil and fat in keratin is not a limiting factor for fungal colonization but it depends much on the nature of fungi. Amount of free amino acids was found maximum i.e., 468±70 μg/ml in culture fluid of *Chrysosporium indicum* grown on
defatted feathers, while maximum amount of protein released was noted
in cultures of *Fusarium oxysporum* when grown on natural feathers (500
µg/ml) and defatted hen feathers (525 µg/ml).

The culture fluids obtained from the cultures of fungi grown on
natural and defatted hen feathers and human hair were also tested for
the presence of lipase, gelatinase and caseinase activity by agar diffusion
method. Culture fluids obtained from *Chrysosporium indicum* (CH3) have
shown maximum lipase activity as compared to other test fungi. Culture
fluids obtained from the culture of *Verticillium* sp. (V1) grown on defatted
and natural human hair showed absence of lipase activity, while its
cultures grown on hen feathers have shown positive lipase activity.
Three test strains including *Chrysosporium indicum* (CH2, CH4) and
*Chrysosporium keratinophilum* (CH6) grown on defatted and natural hairs
and feathers failed to produced extracellular lipase. The absence of lipase
activity in culture fluids of above fungi and production of lipase on agar
medium containing sorbitan monolaurate indicates the inductive nature
of these fungi for lipase synthesis. Gelatinase activity was found in all
the cultures grown on defatted and natural human hair and hen
feathers. Its greater activity was recorded in the cultures of *Fusarium* sp.
(F13), *Microsporum gypseum* (MI2), *Microsporum gypseum* (MI3),
*Trichophyton indicum* (T1) and *Verticillium* sp. (V1). No caseinase activity
was found in culture fluids of *Chrysosporium indicum* (CH2) and
*Chrysosporium keratinophilum* (CH6) when grown on natural and
defatted human hair. In all other cases its activity was found positive.
*Fusarium* sp. (F13) showed maximum activity of caseinase when grown
on human hair, while *Microsporum gypseum* (M12) showed its maximum activity in cultures grown on natural and defatted hen feathers. *Trichophyton* sp. (T1) also showed greater activity of this enzyme in its culture grown on defatted hen feathers.

On the basis of results obtained in previous studies six test fungi i.e. *Chrysosporium indicum* (CH3), *Malbranchea* sp. (MA3), *Microsporum gypseum* (M13), *Fusarium oxysporum* (F7), *Fusarium chlamydosporum* (F8) and *Verticillium* sp. (VI) were selected for further studies. These fungi were tested for their keratin (hen feathers) degrading capabilities (Keratinolysis) in buffered medium at pH 7.

The key reactions in keratinolysis of native keratin are (1) deamination (creating an alkaline environment needed for substrate swelling) (ii) sulphotolysis (denaturing the substrate by removing the characteristic disulphide bridges) and (iii) Proteolysis (cleaving the denatured keratin to soluble products). A concerted action of all three processes results in the production of soluble proteins and amino acids from the native keratin. In view of the above, the studies were planned to determine the change in pH of cultures, release of cysteine and liberation of proteins and free amino acids during the growth of test fungi on defatted hen feathers. In the process of proteolysis, neutral and alkaline proteases seems to play a significant role and hence activity of neutral and alkaline proteases was also determined in the cultures of these fungi. In order to study the effect of incubation period all above tests were performed after an incubation of 10, 20, 30 and 40 days. Amongst the test strains, *Verticillium* sp. (VI) caused maximum weight
loss of feather keratin in 40 days of its growth. *Fusarium oxysporum* was found next to *Verticillium* sp. and caused a total of 71.0±0.5% loss in feather keratin in 40 days. During the growth of all the test fungi on feather keratin in broth cultures, the pH of the culture fluid shifted towards alkalinity. A maximum change in pH i.e., 8.4±0.01 was noted in cultures of *Fusarium oxysporum*, while in cultures of *Fusarium chlamydosporum*, *Malbranchea* sp., *Microsporum gypseum* the final pH was 8.27, 8.27, 8.25, respectively. An increase in the alkalinity confirms the operation of deamination processes during fungal growth on feather keratin. The process considered to be of prime importance in keratinolysis. The amount of free amino acid and cysteine was noted maximum in the cultures of all the test fungi after 40 days of incubation as compared to the cultures tested after 10, 20 and 30 days. The maximum cysteine content was noted in culture of *Verticillium* sp. i.e., 11.25 μg/ml, followed by *Chrysosporium indicum* (5.875 μg/ml), *Fusarium chlamydosporum* (4.25 μg/ml), *Malbranchea* sp. (4.00 μg/ml), *Fusarium oxysporum* (3.5 μg/ml) and *Microsporum gypseum* (2.75 μg/ml). The amount of free amino acids was also found to be maximum in case of *Verticillium* sp. i.e., 510 μg/ml, followed by *Fusarium oxysporum* (470 μg/ml), *Malbranchea* sp. (350 μg/ml), *Microsporum gypseum* (320 μg/ml), *Chrysosporium indicum* (310 μg/ml) and *Fusarium chlamydosporum* (300 μg/ml). The amount of soluble proteins was noted more in 30 days old cultures in almost all the test fungi as compared to their respective 40 days old cultures. A decrease in the amount of protein and an increase
in the amount of cysteine and free amino acid content in 40 days old cultures is not surprising but confirms the proteolysis of the released proteins from the native keratin molecules during later phase of keratin degradation. The presence of activity of neutral and alkaline protease in 10, 20, 30 and 40 days old cultures of all the test fungi along with an increase in cysteine content and a change in pH of the culture fluid confirms a concerted action of different enzymes (keratinases, neutral and alkaline protease) on native keratin.

The keratinase and protease from different sources may differ in their molecular diversity and that the multiple and diverse isozymes from different sources may differ in their total potential to hydrolyze a given substrate. In the present study keratinase activity was assayed in the culture filtrates by using commercially available keratin substrate dyed with Ramazol Brilliant Blue R (Keratin azure). The dye is covalently linked to substrate and release of blue colour is a measure of keratin degradation. Degradation of keratin azure in-vitro leads to the release of blue colour into the medium which can be measured spectrophotometrically at 595 nm. In the present study culture filtrates obtained from 40 days old fungal cultures grown on feathers were used as enzyme sample.

The amount of released dye was determined in the reaction mixture with an interval of 1 hr up to six hours. Enzyme samples obtained from cultures of Verticillium sp. (VI) caused maximum degradation of keratin azure followed by Chrysosporium indicum (CH3) and Malbranchea sp. (MA3). Test strains of Fusarium and Microsporum
showed poor activity of keratinase using keratin azure as substrate. The rate of enzyme activity was found to be more during first hour of reaction in almost all the cases which gradually decreased in case of *F. oxysporum*, *F. chlamydosporum* and *Verticillium* species. However, no definite pattern in rate of enzyme activity was noted in case of all three typical keratinophilic strains (i.e. *Microsporum gypseum*, *Malbranchea* sp. and *Chrysosporium indicum*).

The test fungi have also been tested for the pattern by which they are involved in keratinolysis. For this the human hair has been used as substrate. The way of invasion of hair by three typical keratinophilic fungi used in present study was found to be different than other test saprophytic keratinophilic species, i.e., *Fusarium chlamydosporum*, *F. oxysporum* and *Verticillium* sp. The data obtained from above studies is of immense value for suggesting the proper way of keratin waste disposal. The process of keratinolysis by three non-keratinophilic saprophytic fungi i.e., two species of *Fusarium* and *Verticillium* have been found to be superior over typical keratinophilic species for their use in processing of feathers from poultry waste. Their use is safe to avoid chances of environmental contamination.