Chapter 6

FUNGAL ENZYMES AND KERATIN DEGRADATION
I. Synthesis of some enzymes by keratinophilic fungi
INTRODUCTION

Occurrence of keratinophilic fungi in various habitats rich in organic matter has been reported by a number of workers (Randhawa and Sandhu, 1965; Garg, 1966; Padhye et al., 1967; and Kushwaha and Agrawal, 1976a). The presence of these molds in various habitats indicate the presence of good enzyme equipment in them by virtue of which they could utilize complex organic matter as sole nitrogen and carbon sources. The enzyme producing capability of dermatophytes is known since 1895 while MacFayden observed proteolytic activity in culture fluid of *Trichophyton tonsurans*. Bodin and Lenormand (1901) reported the production of several extracellular enzymes by *Microsporum* sp. Tate (1929) reported the presence of respiratory, proteolytic and lipolytic enzymes including carbohydrases and ureases in dermatophytes. The involvement of several enzymes in the process of infection of *T. mentagrophytes* have been reported by Rippon and Garber (1969). Keeping all these facts in mind the present study was carried out to determine the production of amylase, protease, lipase, urease, chitinase and phosphatase producing capability of some keratinophilic fungi which were of common occurrence in the soil and are also well known dermatophytes. In this experiment the enzyme producing capability of the test organisms was qualitatively evaluated, the detailed study of some enzymes produced by four selected keratinophilic fungi was done in the next experiments.
MATERIALS AND METHODS

The enzyme producing capability of ten keratinophilic fungi (i.e., Auxarthron conjugatum, Chrysosporium crassitunicatum, C. indicum, C. pannicola, C. tropicum, Keratinomyces ajelloi, Malbranchea aurantiaca, Microsporum fulvum, M. gypseum, Trichophyton rubrum and T. terrestris) was assayed on solid media using the method as described by Hankin and Anagnostakis (1975). The media described below were sterilized at 15 lbs pressure for 15 minutes. Twenty ml sterile medium was poured in each Petri plate and inoculated with the mycelial fragments of the test fungus, in the centre of each dish. The dishes were incubated for 5 days at 28°C and enzyme production was assayed as follows:

(i) **Amylolytic activity:**

Amylolytic activity was determined using nutrient agar medium containing 2% soluble starch (pH 6). After 5 days of incubation the plates were flooded with Lugals iodine solution (Society of American Microbiologist, 1957; Kushwaha and Agrawal, 1975) and the nonblued zone around the colony shows the extent of enzyme amylase.

(ii) **Chitinase activity:**

Chitinase activity was determined by using medium containing - 500 ml of mineral salt solution \((\text{NH}_4)_2\text{SO}_4\),
2 g; KH$_2$PO$_4$, 4 g; Na$_2$HPO$_4$, 6 g; FeSO$_4$·7H$_2$O, 0.2 g; CaCl$_2$, 1 mg; H$_3$BO$_3$, 10 ug; MnSO$_4$, 10 ug; ZnSO$_4$, 70 ug; CuSO$_4$, 50 ug; MoO$_3$, 10 ug, Distilled water, 1000 ml), 500 ml distilled water, 0.02% yeast extract, purified chitin 2.4% and agar, 15 g. After 5 days of incubation clear zone in a opaque agar around colonies indicate the presence of enzyme chitinase.

(iii) Lipolytic activity:

The basal medium containing peptone, 10 g; NaCl, 5 g; CaCl$_2$·2H$_2$O, 1 g; Agar, 15 g (pH 6). per liter of distilled water. Tween 20 (Sorbiton monolaurate) was sterilized separately by autoclaving, and 1 ml of this was added in 100 ml of sterilized cooled basal medium as lipid substrate.

The lipase activity was determined after 5 days of incubation by observing visible precipitate due to the formation of crystals of calcium salt of the lauric acid liberated by the enzyme, or a cleaning of such precipitate around a colony due to the complete degradation of the salts of the fatty acids.

(iv) Phosphatase activity:

For this coconut plate agar composed of 98 ml (pH 8) sterile cooled medium plus 2 ml of sterile 0.01 M phenolphthalein diphosphate sodium salt were used. After 5 days of incubation the plates were opened and inverted over a
container of NH₄OH. The colonies that turn pink to red were presumed to have degraded the substrates.

(v) **Proteolytic activity**

The medium consisting of nutrient agar plus 4% gelatin (pH 6) was used. Solution of gelatin (8%) in water was sterilized separately and added to the nutrient agar at the rate of 5 ml per 100 ml of medium. After 5 days of incubation complete degradation of gelatin was seen as clear zone in opaque agar around colonies indicate the presence of an enzyme protease. The plates were then flooded with aqueous saturated solution of ammonium sulphate, a precipitate was formed which make the agar more opaque and enhanced the clarity of the zone around the colony.

(vi) **Urease activity**

For this coconut agar (pH 6) plus 1% urea were used. Urea solution of 0.5% per ml was prepared and sterilized by millipore filtration. Two ml of sterilized urea solution was added to each 100 ml.

After 5 days of incubation urease production was detected by pouring an over layer containing urea and indicator over the surface. The over layer contained 100 ml of 0.01 M phosphate buffer (pH 6), 1.5 g agar and 1 g
urea. The urea was added after a liquified buffer mixture
has been cooled to preclude hydrolysis. Before pouring,
.5 ml of a 1% solution of bromthymol blue was added. The
colour of over layer should be orange to yellow (if green
0.1 N HCL was added to adjust the colour). The urease
production is determined by turning over layer into blue.
At least two hours should be allowed before recording a
negative result.

RESULTS AND DISCUSSION

This experiment was performed only to evaluate
whether the test organisms showed negative or positive
activity for various enzymes under study. The detailed
study of some of these enzymes has been done in further
experiments. The data showing ability of keratinophilic
fungi to synthesize various enzymes on solid media are
shown in Table 21. A perusal of the data clearly indicates
that all the fungi were able to produce enzyme amylase.
The crystals of calcium salt of lauric acid were also
formed in all the cases indicating the production of lipase.
When culture dishes were flooded with aqueous ammonium
sulphate solution the opaque clear zone around the colony
were formed due to proteolytic activity. Phosphatase
activity was also recorded in all the cases. Chitinase
producing capability was found in A. conjugatum, C. tropicum,
K. ajelloi, M. fulvum, M. gypseum, T. rubrum and T. terrestre.
<table>
<thead>
<tr>
<th>Organisms</th>
<th>Amylase</th>
<th>Lipase</th>
<th>Protease</th>
<th>Chitinase</th>
<th>Urease</th>
<th>Phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Auxarthron conjugatum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chrysosporium crassitunicatum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chrysosporium indicum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chrysosporium pannicola</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chrysosporium tropicum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Keratinomyces ajelloi</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Malbranchea aurentiaca</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Microsporum fulvum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Microsporum gypseum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trichophyton rubrum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trichophyton terrestre</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

( + ) indicates presence of enzyme.
( - ) indicates absence of enzyme.
With the exception of *C. tropicum* and *M. aurantiaca* all the test fungi could hydrolyse urea.

The production of various enzymes by keratinophilic fungi is of immense value as these are responsible for keratinolysis in nature and also during the infection to man and animals by invading the keratinized tissues of skin. The present study also confirms the saprophytic survival of these molds in various habitats in nature. These investigation are also sufficient to disclose the association of such molds with pathogenic attack and having wide distribution.
II. Production of Amylase
INTRODUCTION

Starch is an excellent carbon source for fungi and actinomycetes (Cochrane, 1958). It is a hexose polysaccharide existing in two forms:

(i) Amylase: It is a linear structure built up to 200 to 500 or more glucose units linked together by 1-4 glycoside bonding.

(ii) Amylopectin: In this individual glucose units are likewise bonded together by 1-4 linkage but the molecules in branches are attached through 1-6 glycoside linkages.

Starch generally contains 70 to 80% amylopectin and 20-30% amylase.

The enzyme which is responsible for the hydrolysis of starch is amylase, also produced extracellularly and remain in the culture filtrate even after harvesting the microorganism, of which the end product of hydrolysis is glucose. The reports on amylase producing capabilities of keratinophilic fungi are very scanty (Sen, 1964; Agrawal and Rao, 1974; Kushwaha and Agrawal, 1975; Jain, 1977; and Singh and Agrawal, 1981a). Keeping above studies in mind an attempt is being made during present investigations to study the production of an enzyme-amylase in relation to incubation period.
MATERIALS AND METHODS

In the present study four fungi (i.e., A. conjugatum, C. pannicola, K. jellouij and M. gypseum) were cultured in two basal media having following composition:

Medium I (Starch broth medium):

Soluble starch 5 g; Yeast extract 2 g; MgSO₄·7H₂O 0.5 g; KH₂PO₄ 1 g; Microelement solution 2 ml (1 ml contained Fe as Fe(NH₄)₂(SO₄)₃ 1 mg; Zn as ZnSO₄ 1 mg and B as H₃BO₃ 0.1 mg) and Distilled water 1000 ml.

Medium II:

Starch broth medium supplemented with 0.2% glucose and 0.1% asparagine.

Erlenmeyer's flasks containing 30 ml of basal medium were sterilized at 15 lbs pressure for 15 minutes and inoculated by 8 mm disc obtained from 10-day old colony of test organisms grown on Sabouraud's dextrose agar medium. Inoculated flasks were incubated at 28°C. A set of flasks without inoculum was also kept as control. After 5, 10, 15 and 20 days of incubation a set of flasks for each organism was removed and filtered through the preweighed filter paper. Dry weight of the mycelium was determined after drying the filter paper at 80°C for 24
hours. The culture filtrate was centrifuged at 10000 rpm for 15 minutes and supernatant was used as crude enzyme sample. The enzyme activity was assayed by the method suggested by Fergus (1969). A cavity (8 mm diameter) made with the help of sterile cork borer in the Petri dish and 2 ml of cell-free culture filtrate was poured. The medium contained in the Petri dishes comprised following ingredients:

Soluble starch 10 g; Na$_2$HPO$_4$ 2.84 g; NaCl 0.35 g; Agar 20 g; and Distilled water 1000 ml.

The assay plates having the filtrates in the cavity were kept for 24 hours at 30°C. The amylase activity was determined by flooding Lugol's Iodine Solution (Society of American Microbiologist, 1957). The activity was determined by measuring the width of nonblued zone around the cavity. An average of three replicates was taken for each organism.

RESULTS AND DISCUSSION

A perusal of the Table 22 showed that most of the keratinophilic fungi used, were able to synthesize enzyme amylase. The maximum dry weight of the mycelium was recorded after 10 days of incubation in almost all the fungi. The medium supplemented with glucose and asparagine
Table - 22: Amylase activity as shown by Iodine Test

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Days of incubation</th>
<th>Medium I</th>
<th></th>
<th>Medium II</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mycelial dry weight (mg)</td>
<td>Width (mm) of non-blued zone</td>
<td>Mycelial dry weight (mg)</td>
<td>Width (mm) of non-blued zone</td>
</tr>
<tr>
<td>A. conjugatum</td>
<td>5</td>
<td>43</td>
<td>-</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>65</td>
<td>5</td>
<td>72</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>60</td>
<td>4</td>
<td>61</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>46</td>
<td>2</td>
<td>44</td>
<td>1.0</td>
</tr>
<tr>
<td>C. pannicola</td>
<td>5</td>
<td>76</td>
<td>-</td>
<td>78</td>
<td>-</td>
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<td></td>
<td>10</td>
<td>109</td>
<td>6</td>
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<td>20</td>
<td>71</td>
<td>2.5</td>
<td>74</td>
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<td>K. ajelloi</td>
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<td>51</td>
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<td></td>
<td>10</td>
<td>88</td>
<td>5</td>
<td>100</td>
<td>4.5</td>
</tr>
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<td></td>
<td>15</td>
<td>80</td>
<td>4</td>
<td>82</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>72</td>
<td>2</td>
<td>78</td>
<td>-</td>
</tr>
<tr>
<td>M. gypseum</td>
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<td>55</td>
<td>-</td>
<td>58</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>72</td>
<td>7</td>
<td>97</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Each datum given in the table is an average of three replicates.
FIG. 8:—AMYLASE ACTIVITY OF KERATINOPHILIC FUNGI IN DIFFERENT MEDIA.
enhanced the growth of almost all the fungi than in starch medium. However, more enzyme activity in latter may be due to the repressive action of glucose (Bilgrami and Verma, 1981). The decrease in the dry weight and amylase activity after 15 and 20 days of incubation may be due to the synthesis of lytic enzymes. The amylase producing capability of some plant pathogenic fungi, thermophilic fungi and some mesophilic saprophytes have been reported by various workers (LeMense et al., 1947; Fergus, 1969; Chaturvedi and Agrawal, 1974; Adam and Deploy, 1976; Schellart et al., 1976; Mangallam et al., 1977; Manjunath and Raghavendra Rao, 1979). Some workers have reported the production of enzyme in submerged culture by Chrysosporium tropicum, Malbranchea pulchella, Trichophyton rubrum and T. mentagrophytes (Sen, 1964; Kushwaha and Agrawal, 1975). A perusal of the literature showed that the soil inhabiting keratinophilic fungi are not only restricted to protein and keratin but may also grow and digest plant debris. The capability of these fungi to produce amylase is important from the point of view of their saprophytic survival in soil.
III. Production of Lipase
INTRODUCTION

Fats occur widely as reserve materials in higher plants, mostly stored in seeds. The fat is also one of the major constituents of the hair, wool and feathers which is supposed to provide the resistance for microbial attack (Bose, 1964; Baxter and Trotter, 1969; Pugh and Evans, 1970). The degradation of fats begins with the breakdown of neutral fats into glycerol and fatty acids. This is brought about by lipases and hydrolytic enzymes belonging to the group of esterases. The product of their degradation can be again subjected to the biological oxidation. They can also be used for synthesis as in glyoxalate cycle for synthesis of glucose. Several fungi are known to produce lipases (Fiore and Nord, 1950; Yamada et al., 1962; Somkuti and Babel, 1968; Somkuti et al., 1969; Arima et al., 1972; Hankin and Anagnostakis, 1975; Adam and Deploey, 1978). Lipase producing capability of dermatophytes have also been reported by many workers (Tate, 1929; Das Gupta and Shome, 1960; Bohme, 1968). The qualitative estimation of this enzyme has been evaluated in previous experiments. In the present investigation the quantitative estimation of lipolysis by four keratinophilic fungi have been determined.
MATERIALS AND METHODS

In the present study production of lipase by four test fungi, i.e., *A. conjugatum*, *C. pannicola*, *K. ajelloi* and *M. gypseum* have been evaluated. The production of lipase was determined following the method of Somkuti and Babel (1968), for this 30 ml wheat bran broth medium (pH 6) was dispersed in each 150 ml Erlenmayer flask and sterilized at 15 lbs pressure for 15 minutes. The flasks were inoculated with a 10 mm disc taken from 2-week old culture grown on Sabouraud's dextrose agar medium and were incubated at 30°C. After 10 days of incubation the contents were filtered, centrifuged at 10000 rpm for 10 minutes and supernatant was used as crude lipase preparation. For determining mycelial weight the filter papers were dried at 80°C.

**Enzyme assay:**

The reaction mixture was prepared in the following manner: 1 g of substrate (butter fat/coconut oil) 0.4 g of Na taurocholate, 1 ml of 0.1 M CaCl₂ and 6 ml of 0.1 M acetate buffer (pH 6) was homogenised at 60°C for 20 min in a waring blender. Three ml of crude enzyme solution and the substrate emulsion were combined in 150 ml flask, the assay mixture was incubated for two hours on reciprocal shaker at 35°C in water bath. At the end of incubation
40 ml of absolute ethyl alcohol was added to stop the reaction and to dissolve the liberated fatty acids. The released fatty acids were titrated with alcoholic KOH 0.02 N using phenolphthalein as an indicator. The control was an assay mixture with boiled enzyme. The difference between the volume of alkali used for titrating an assay mixture, the Control was taken as the amount of free fatty acids liberated during the incubation.

RESULTS AND DISCUSSION

The solution required for titration of liberated fatty acids was 8.8, 6.2, 11.6 and 12.4 volume of 0.02 N KOH in case of *A. conjugatum*, *C. pannicola*, *K. ajelloi* and *M. gypseum*, respectively, when butter fats was used as a substrate (Table 23). In coconut oil the volume of alcoholic 0.02 N KOH required for titration of liberated fatty acid by the action of lipase of *A. conjugatum*, *C. pannicola*, *K. ajelloi*, *M. gypseum* was 7.4, 4.3, 10.8 and 8.4, respectively. *M. gypseum* had been already shown to possess the lipolytic ability (Bohme, 1968). The data indicate that the nature of substrate (butter fat/coconut oil) influence the enzyme activity. The well known dermatophytes, viz., *K. ajelloi* and *M. gypseum* have more lipolytic activity than other test organisms indicating the role of this enzyme during pathogenesis. The production of lipase by all the
Table - 23: Lipolysis of butter fat and coconut oil by lipase from some keratinophilic fungi in wheat bran broth after 10 days.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Dry mycelial weight (mg)</th>
<th>Volume of 0.02 KOH required for titration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Butter fat</td>
</tr>
<tr>
<td><em>A. conjugatum</em></td>
<td>62</td>
<td>8.8</td>
</tr>
<tr>
<td><em>C. pannicola</em></td>
<td>109</td>
<td>6.2</td>
</tr>
<tr>
<td><em>K. ajelloi</em></td>
<td>86</td>
<td>11.6</td>
</tr>
<tr>
<td><em>M. gypseum</em></td>
<td>72</td>
<td>12.4</td>
</tr>
</tbody>
</table>

Each datum given in the table is an average of three replicates.
test fungi is also important from the point of view of their saprophytic survival in soil.
IV. Production of Protease
INTRODUCTION

Microorganisms are known to produce proteolytic enzymes for the hydrolysis of protein into polypeptides and amino acids (Hankin et al., 1976). Based on the size of the molecule that they can attack, or preferably attack proteolytic enzymes are called (a) proteinases when they hydrolyse proteins to polypeptides, (b) peptidases, when they hydrolyse polypeptides into smaller peptides and amino acids. The distinction between the proteinases and peptidases is not very realistic (Agrios, 1969). The term proteases refers to a mixture of proteolytic enzymes which may include proteinases and peptidases (Wallerstein, 1939).

Protease production by dermatophytes is known since the time of MacFayden (1895), he observed the proteolytic activity in the culture fluid of Trichophyton tonsurans. Later on Tate (1929), Fujii (1955), Kunert (1970) and Mevootisom and Niederpruem (1979) have reported the production of protease by dermatophytes. The protease along with keratinase production by the members of this special group of fungi is important for medical mycologist because these are always associated with the mycotic infections of keratinized tissues of man and animals and produced in a wide range of pH (Cruickshanke and Trotter, 1956; O'Sullivan and Mathison, 1971). The present study was planned to
evaluate the extracellular production of proteases by four keratinophilic fungi, i.e., *A. conjugatum*, *C. pannicola*, *K. ajelloi* and *M. gypseum* in relation to different proteinaceous substrates.
MATERIALS AND METHODS

In the present study all the test fungi (A. conjugatum, C. pannicola, K. ajelloi and M. gypseum) were cultured in glucose gelatin medium of the following composition:
Gelatin 10 g; Glucose 10 g; K$_2$HPO$_4$ 1 g; MgSO$_4$.7H$_2$O 0.5 g; Distilled water 1000 ml.

To evaluate the nature of enzyme following basal media were used:

(i) Glucose asparagine broth:
    Asparagine 2 g; K$_2$HPO$_4$ 1 g; MgSO$_4$.7H$_2$O 0.5 g;
    Dextrose 10 g; Distilled water 1000 ml (pH 6.9).

(ii) Gelatin broth:
    Gelatin 10 g; K$_2$HPO$_4$ 1 g; MgSO$_4$.7H$_2$O 0.5 g
    and Distilled water 1000 ml.

(iii) Casein broth:
    Casein 10 g; K$_2$HPO$_4$ 1 g; MgSO$_4$.7H$_2$O 0.5 g and
    Distilled water 1000 ml.

(iv) Soya bean meal broth:
    NaNO$_3$ 2 g; K$_2$HPO$_4$ 1 g; MgSO$_4$.7H$_2$O 0.5 g;
    KCl 0.5 g; FeSO$_4$.7H$_2$O 0.01 g; Soyabean meal
    30 g and Distilled water 1000 ml.
Basal medium (30 ml) was taken into each 150 ml Erlenmeyer's flask and sterilized at 15 lbs pressure for 15 minutes. The flasks were inoculated with 10 mm inoculum disc taken from 2-week old culture grown on Sabouraud's dextrose agar medium. The flasks were incubated at 28 ± 1°C. The content was filtered through pre-weighed Whatman No. 1 filter paper after 10 days. Mycelial dry weight was recorded in each case. The culture filtrate was centrifuged at 10,000 rpm for 15 minutes and supernatant was taken as crude enzyme preparation.

**Enzyme assay:**

The enzyme activity was determined by a modified method of Hayashi et al. (1967) as also followed by Mayers Ahearn (1977). The reaction mixture contain 0.5 ml cell-free culture broth, 0.5 ml McIlvaine's citrate buffer (pH 6.0). The boiled enzyme sample was added in reaction mixture for control. The mixture was then incubated for 20 minutes at 35°C and the reaction was stopped by adding 4.0 ml of 5.0% trichloroacetic acid. After an hour the solution was passed through Whatman No. 1 filter paper. One ml of filtrate was mixed with 5.0 ml of 0.4 u Na₂CO₃ followed by addition of 0.5 ml phenol reagent as described by Lowery et al. (1951). The liberation of tyrosine was determined with Carl/Zeiss/Jena Spekol at 660 nm. The
amount of tyrosine thus determined was calculated by multiplying it with 6 (as total volume of the filtrate was 6 ml) and divided by 20 in order to calculate the amount of tyrosine liberated per 0.5 ml of culture broth per minute. A unit of an enzyme activity was defined as an amount of enzyme liberating one micromole of tyrosine per minute under defined condition.

RESULTS AND DISCUSSION

A perusal of the data (Table 24) indicate that all the test fungi could produce extracellular protease. The recorded enzyme units were 4.87, 4.62, 5.89 and 5.57 in case of A. conjugatum, C. pannicola, K. ajelloi and M. gypseum, respectively, in glucose gelatin medium. Maximum production of enzyme was recorded when soyabean was provided as protein source. The production of enzyme by these molds varies from substrate to substrate. It is interesting to note that a little synthesis of protease was also noticed in the presence of glucose asparagine medium which lack the protein source. The production of an enzyme even in the absence of any protein source indicates the constitutive nature of enzyme. However, comparatively the enzyme production is much less in this medium than glucose gelatin medium.

Various workers (Bohme and Ziegler, 1967; Kunert, 1970;
Table - 24 : Production of protease by keratinophilic fungi in different broth media having various protein sources.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Glucose gelatin</th>
<th>Glucose asparagine</th>
<th>Gelatin</th>
<th>Casein</th>
<th>Soyabean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mycelial Tyrosine units</td>
<td>Mycelial Tyrosine units</td>
<td>Mycelial Tyrosine units</td>
<td>Mycelial Tyrosine units</td>
<td>Mycelial Tyrosine units</td>
</tr>
<tr>
<td><strong>Auxarthron conjugatum</strong></td>
<td>148</td>
<td>4.87</td>
<td>128</td>
<td>2.0</td>
<td>81</td>
</tr>
<tr>
<td><strong>Chrysosporium pannicola</strong></td>
<td>62</td>
<td>4.62</td>
<td>73</td>
<td>1.6</td>
<td>58</td>
</tr>
<tr>
<td><strong>Keratinomyces sieloi</strong></td>
<td>80</td>
<td>5.89</td>
<td>74</td>
<td>2.8</td>
<td>51</td>
</tr>
<tr>
<td><strong>Microsporum gypseum</strong></td>
<td>59</td>
<td>5.57</td>
<td>82</td>
<td>2.5</td>
<td>43</td>
</tr>
</tbody>
</table>

Each datum given in the table is an average of three replicates.
FIG. 9:— PROTEASE PRODUCTION IN BROTH MEDIUM CONTAINING DIFFERENT PROTEIN SOURCES.
and Singh (B.G.), 1981) have indicated that there is no direct correlation between the proteolytic activity and the keratin digestion. The pH also affects the proteolytic activity. This enzyme remains active over a wide range of pH. This property is likely to be encountered in the parasitic environment and is appeared to be necessary for acquiring the successful proteolytic attack of the skin and its appendages by dermatophytes (Subrahmanyan, 1980). Minocha et al. (1972) have shown proteolytic activity and its role in pathogenesis of dermatophytes. The production of protease by these molds is also important from the point of view of saprophytic survival of these molds in the soil.
V. In vitro Degradation of Human Hair
INTRODUCTION

Keratin, the fibrous protein is a cornified part of the skin of vertebrates and includes hair, wool and related structures, which differ from other proteins in their high cystine content. The cystine molecules with disulphide linkages are thought to be responsible for providing stability to the keratin molecule and rendering it more resistant to enzymatic digestion. The manner in which the dermatophytes attack and digest this very resistant substrate is one of the central problems of the physiology of keratinophilic moulds. The dermatophytes can grow even on the hardest keratin as the only source of carbon and nitrogen. The ability of soil inhabiting keratinophilic fungi to digest naturally occurring keratin molecules has prompted several investigations. The digestion is accompanied by the synthesis of a large number of enzymes, particularly proteolytic and keratinolytic ones (Weary et al., 1965; Yu et al., 1968). The breakdown of keratin into proteins, peptides and amino acids results in alkalinization of the medium.

The ability of five keratinophilic fungi and related dermatophytes to degrade human hair is studied in the present investigation.
MATERIALS AND METHODS

Preparation of keratinic substrates:

Hair were obtained from a 22 years old male. After washing four times in chloroform and four times in water the samples were air dried. The hair were cut into pieces of about 2 cm length, weighed into portions of 500 mg and then sterilized in propylene oxide for 24 hours.

Preparation of fungal inoculum:

Four fungi, i.e., Auxarthron conjugatum, Chrysosporium pannicola, Keratinomyces ajelloi and Microsporum gypseum were used as test organisms. The fungi were grown on Sabouraud's dextrose agar medium. To prepare the inoculum spores of each test organism were obtained and a suspension was prepared in a sterile basal salt solution. The density of spores in the suspension was controlled by a haemocytometer and adjusted to a concentration of \(10^6\) spores in one ml.

Inoculation of flasks:

A basal solution was employed which contained the following salts: \(K_2HPO_4\) 1.5 g, \(MgSO_4\cdot7H_2O\) 0.025 g, \(CaCl_2\) 0.025 g, \(FeSO_4\cdot7H_2O\) 0.015 g, \(ZnSO_4\cdot7H_2O\) 0.005 g per litre. Erlenmeyer flasks containing 45 ml of the sterile basal salt solution were divided in three sets as follows:
**Keratin control:** To this were added 500 mg of sterile hair and 5 ml of the sterile basal salt solution.

**Fungus control:** To this were added 5 ml of the fungal inoculum.

**Test samples:** To this were added 500 mg of hair and 5 ml of fungal inoculum.

All the flasks were cultured on a rotatory shaker at 300 rpm at 27 ± 2°C for different periods of time.

**Determination of keratinolytic activity:**

From each set one flask was removed after weekly intervals starting from one week till six weeks. The culture fluid was centrifuged and the supernatant was assayed as follows:

**Release of protein:** The protein was estimated by the method described by Lowry *et al.* (1951) using bovine serum albumin as standard. Experimental values are expressed as ug of proteins per ml of supernatant.

**Sulphydryl compounds:** The determination of SH compounds was carried out colorometrically according to the method of Saville (1958).

**Concentration of hydrogen ions:** After each interval of pH
of the suspension was measured with the help of a digital pH meter 335.

**Substrate decomposition:** The rate of hair decomposition was determined using the method described by Chesters and Mathison (1963).

**Structural damage:** The structural damage in hair samples was noted by light microscopy (x 100).

The data presented in Fig. 1 are expressed as net values of protein and soluble sulphhydril groups, i.e., the measured value in the test flask minus the sum of the values of keratin and fungal controls.

**RESULTS AND DISCUSSION**

The net protein released from hair and the pH of the culture fluid over the experimental period are given in Fig. 10 and Table 25. Most rapid release of protein was observed after 3 weeks of incubation in each case. The highest value of released protein, i.e., 86 ug/ml was obtained after 3 weeks with *M. gypseum*, followed by *C. pannicola* (81 ug/ml), *K. jellolo* (74 ug/ml) and *A. coniugatum* (55 ug/ml). The same pattern was seen when the percentage of weight loss of the hair samples was taken as parameter of fungal keratinolytic activity (Table 26). It is quite apparent from the data that in all cultures the
FIG. 10: NET PROTEIN RELEASED INTO THE MEDIUM DURING GROWTH OF TEST ORGANISMS ON HUMAN HAIR.
Table 25: Change in pH during growth of keratinophilic fungi on human hair in shake cultures.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Time of incubation (week)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>A. conjugatum</td>
<td>7.41</td>
</tr>
<tr>
<td>C. pannicola</td>
<td>7.52</td>
</tr>
<tr>
<td>K. aielloi</td>
<td>7.45</td>
</tr>
<tr>
<td>M. gypseum</td>
<td>7.57</td>
</tr>
</tbody>
</table>

Each datum given in the table is an average of three replicates.
Table - 26: Weight loss (%) in hair samples following fungal degradation after 6 weeks of incubation

<table>
<thead>
<tr>
<th>Organism</th>
<th>Loss in weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Auxarthron conjugatum</td>
<td>19.6</td>
</tr>
<tr>
<td>Chrysosporium pannicola</td>
<td>27.91</td>
</tr>
<tr>
<td>Keratinomyces aielloi</td>
<td>25.57</td>
</tr>
<tr>
<td>Microsporum gypseum</td>
<td>29.5</td>
</tr>
</tbody>
</table>
Fig. 1: Degradation of human hair by *Microsporum gypseum* showing disruption, X 100

Fig. 2: Degradation of human hair by *Keratinomyces ajelloi* showing disruption, X 100
pH increased toward higher alkalinity as the incubation period advanced up to 4 weeks. However, a decrease in alkalinity of the culture media was noted after further progress of the incubation time, i.e., after four weeks. The quantitative relation of the effects of the different fungi during the process of hair digestion as suggested by the release of protein is also reflected in the pH measurements. The level of sulphhydryl groups in the culture media was quite low throughout the experiment. The microscopic examination of hair samples showed extensive hair damage with *M. gypseum* (Plate 15, Fig. 1) and *K. ajelloi* (Plate 15, Fig. 2). *Chrysosporium pannicola* produce extensive rupturing and bristling of hair cuticle but *A. conjugatum* could cause only minimal disruption. The degradation of hair may be due to the synthesis of enzyme(s), keratinase(s) which are proteolytic in nature. Yu et al. (1968, 1969, 1971) have given evidence, that only keratinase is able to digest hair. In the present investigation, *M. gypseum* showed the highest and *A. conjugatum* the lowest keratinolytic activity.