Characterization of Keratinase
3. CHARACTERISATION OF KERATINASE

3.1. INTRODUCTION

Keratinolytic fungi produces different complex of proteolytic enzymes (Asahi et al. 1985; Sanyal et al. 1985; Tsuboi et al. 1987 & 1989; Apodaca Mc Kerrow 1990; Grzywnowicz et al. 1989). In T. rubrum, for example, electrophoresis indicated the presence of enzymes with different molecular weights. (Asahi et al. 1985; Apodaca and Mc Kerrow 1989a & 1989b). Proteolytic enzymes of keratinophilic fungi have a broad range of specificity, which can hydrolyze both soluble and insoluble proteins.

Extracellular enzymes of dermatophytes and soil inhabiting keratinophilic fungi belong to neutral or alkaline proteases. The suitable pH for their optimum activity varies in media containing different proteins and ranges between pH 6 to 9. With insoluble proteins, particularly keratin, the values usually approach the upper limit. In exceptional cases, optimum values of pH 9 to 10 were found even on soluble proteins (Day et al. 1968; O’Sullivan and Mathison 1971; Takiuchi et al. 1982 & 1984). Though different proteases of keratinophilic fungi were purified to homogeneity, (Ruffin et al. 1979; Asahi et al. 1985; Apodaca and Mc Kerrow 1989a & 1989b; Sanyal et al. 1985; Rajak et al. 1991; Qin et al. 1992; Lambkin et al. 1994; Ferreiro 1997; Aubaid and Muhsin 1998; Anbu 2008) their classification within the international system of enzymes remains mostly uncertain. According to their characteristics (e.g. the alkaline optimum pH) the proteases of dermatophytes were described first as trypsin-like and later (according to
their specificity) as chymotrypsin-like. Recently, purified enzymes are mostly sensitive to class-specific inhibitors of serine proteases. They are also often inhibited by chelating agents (e.g. EDTA), which may be due to the sequestration of Ca\textsuperscript{2+} ions. Calcium ions are frequently bound by serine proteases and increase their activity and stability. Many fungal proteases belong to the class of metalloproteases and consequently the production of metallo enzymes is also highly probable in keratinophilic fungi. Recent studies have revealed that many fungal “chymotrypsin-like” or “elastase-like” enzymes are in fact members of the subtilisin subfamily of serine proteases (Ohman \textit{et al.} 1996; Monod 2008; Anbu \textit{et al.} 2008).

Thus, it is important to study the characteristics of these enzymes such as pH temperature and its molecular weight to understand the action better. In the present study in addition to the activity profiling of the keratinase, optimum temperature and pH needed for its activity were also been studied.

### 3.2. METHODOLOGY

#### 3.2.1. pH AND KERATINASE ACTIVITY

\textit{(Ashai \textit{et al.} 1985 & Gradisar \textit{et al.} 2005)}

The keratinase activity of the crude enzyme extract at different pH was measured. 500 µl of the crude enzyme extract solution was transferred to a sterile eppendorf tube containing 1% (w/v) keratin powder (Hi-media) in 20 mM of different buffer at different pH and incubated at room temperature for 5 hr for the enzyme digestion. Citric acid-disodium phosphate buffer was used for pH 6; Tris-HCl Buffers was used for pH 7 & pH
8 and Glycine–NaOH buffer for pH 9 & pH 10. Keratinase enzyme activity was assayed by Folin-Ciocalteu method mentioned earlier. The keratinase activity profile at different pH was assessed for each clinical isolates of each strain.

3.2.2. TEMPERATURE AND KERATINASE ACTIVITY
(Raju et al. 2007)

The keratinase activity of the crude enzyme extract at different temperature was measured. 500 µl of the crude enzyme extract solution was transferred to a sterile eppendorf tube containing 1% (w/v) keratin powder (Hi-media) in 20 mM Tris-HCl buffer (pH 8.0). And the tube was incubated for enzyme digestion for 5 hours at different temperatures. water bath incubator (Remi, India) was used to maintain different temperatures such as 25, 30, 35, 40, 45 and 50°C. Keratinase enzyme activity was assayed by Folin-Ciocalteu method mentioned earlier. The keratinase activity profile at different temperature was assessed for each clinical isolates of each strain.

3.2.3. PARTIAL PURIFICATION OF KERATINASE
(Asahi et al. 1985; Bressollier et al. 1999)

Six ml of the crude enzyme extract was taken in a 25 ml falcon tube and the proteins present in the extract were precipitated by adding 14 ml (70%) of saturated Ammonium Sulfate. After mild vortexing, the solution was centrifuged at 20000x g for 20 min. The precipitate was collected and dissolved in 4 ml of distilled water. Amicon Ultra-4 filter with 10 kDa NMWL was used for desalting and concentration. The reconstituted sample was added to the Amicon filter reservoir. This filter assembly (collection tube and filter
reservoir) was centrifuged at 4000x g in a swinging bucket rotor for 10 min. First filtrate collected in the collection tube was removed and set aside. Once again water was added to the filter device to bring the sample volume up to 4 ml, centrifuged again and the filtrate was collected and kept aside. Same way the sample was washed once again with 4 ml water. The three filtrates were kept aside till the sample was fully recovered and analyzed. The concentrated sample was dissolved in 3 ml of 150 mM NaCl/ 20 mM-Tris-HCl buffer, pH 8. This enzyme concentrate solution was applied to a Sephadex G-100 column (1.9 cm x 110 cm). Fractions were eluted at a flow rate of 15 ml/ hr using same buffer pH 8 and 2 ml fractions were collected each time. The fractions were checked for keratinase enzyme activity. Fractions, which showed keratinase activity were pooled, precipitated once again using Ammonium Sulphate precipitation method and the precipitate was desalted and concentrated as per the protocol mentioned above, which was used for SDS-PAGE run.

3.2.4. MOLECULAR WEIGHT MEASUREMENT OF KERATINASE
(Heussen and Dowdle 1980; Apodaca and McKerrow 1989b; Jousson et al. 2004a & 2004b)

SDS- Poly acrylamide gels were used to detect the Molecular Weight (MW) of the partially purified keratinase enzyme fraction. Slab SDS- polyacrylamide gel of 8 x 7 cm x 0.75 mm thickness with the composition of 10% resolving and 4% stacking gel was prepared on a slab gel apparatus (Helini- mini slab gel apparatus & Mini power pack). Purified samples of each isolate of each strain were diluted with an equal volume of 2x sample buffer (0.25 M Tris hydrochloride [pH 6.8], 20% [v/v] glycerol, 0.2% [w/v]
sodium dodecyl sulfate, and 0.005\% [w/vl] bromophenol blue). 10 to 15 \( \mu l \) of the above sample mixture was loaded on the gel. A pH 8.3 Tris/ Glycine buffer was used as running buffer. Electrophoresis was run at 20 mA/ gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 in water-methanol-acetic acid (5:5:1, v/v) for 30 min and then destained in 45\% (v/v) methanol/ 3\% (v/v) acetic acid until the bands are clearly visible for photography. For calculation of MW molecular weight standards viz. 116.00 kDa, 66.00 kDa, 45.00 kDa, 35.00 kDa, 25.00 kDa, 18.40 kDa, 14.40 kDa were run simultaneously in a separate lane of the gel. Using Genetools software (PerkinElmer, USA) the MW of the digested peptide fragments was determined.

3.3. RESULTS

3.3.1. pH AND KERATINASE ACTIVITY

Keratinase enzyme activity of the different strains at different pH was studied. T. rubrum showed a peak keratinase activity of 19.48 KU at pH 8 and was found to increase from pH 7 (14.73 KU) and reached the peak activity at pH 8 and remained moderate till pH 9 (13.40 KU). M. gypseum showed a peak keratinase activity of 25.65 KU at pH 8. The keratinase activity of M. gypseum remained close to higher level till pH 9 (22.30 KU). M. canis showed a peak keratinase activity (15.42 KU) at pH 8. The keratinase activity was at moderate level from pH 7 (11.55 KU) and reached the peak activity at pH 8 and remained in moderate level till pH 9 (12.37 KU). Keratinase activity of E. floccosum was at moderate level at pH 7 (9.08 KU). E. floccosum recorded a peak activity of 10.13 KU at pH 8 and it dropped to 7.75 KU at pH 9 (Graph-19).
3.3.2. TEMPERATURE AND KERATINASE ACTIVITY

Keratinase activity of the strains at different temperatures was studied. It was noted that the keratinase activity of all the dermatophytes were found to be higher at 30°C - 40°C. *T. rubrum* recorded peak keratinase activity of 15.42 KU at 35°C. *M. gypseum* showed a peak keratinase activity of 25.65 KU at 35°C. *M. canis* showed the peak keratinase activity of 19.18 KU at 35°C, while *E. floccosum* recorded peak keratinase activity of 11.55 KU at 35°C (Graph-20).
3.3.3. MOLECULAR WEIGHT (MW) OF KERATINASE

The crude enzyme extract of the isolates of each species were partially purified individually and the active fractions, which showed keratinase activity were pooled together and run through gel electrophoresis to find out the molecular weight of the enzyme. This experiment recorded that *T. rubrum* isolates showed an active fragment with a mean MW 36.21 kDa. *M. gypseum* isolates showed an active fragment with a mean MW of 33.19 kDa, whereas *M. canis* isolates showed an active fragment with a mean MW of 33.26 kDa. Isolates of *E. floccosum* showed an active fragment with a mean MW of 31.41 kDa (Fig. 15, 16, 17 & 18; Table-8).

Table 8: Molecular Weight (MW) of keratinase produced by different isolates of dermatophytes after purification

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Molecular Weight (MW) of the Isolates in kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>T. rubrum</em></td>
</tr>
<tr>
<td>1</td>
<td>36.73</td>
</tr>
<tr>
<td>2</td>
<td>37.09</td>
</tr>
<tr>
<td>3</td>
<td>36.73</td>
</tr>
<tr>
<td>4</td>
<td>36.03</td>
</tr>
<tr>
<td>5</td>
<td>35.34</td>
</tr>
<tr>
<td>6</td>
<td>35.34</td>
</tr>
<tr>
<td>Mean MW</td>
<td>36.21</td>
</tr>
</tbody>
</table>
Fig 15: Molecular Weight of Keratinase purified from *E. floccosum* isolates

Track - 1, 2, 3, 5, 6, 7 are partially purified keratinase from isolates of *E. floccosum*

Track - 4: Protein MW Standards

Fig 16: Molecular Weight of keratinase purified from *T. rubrum* isolates

Track - 1, 2, 3, 4, 6, 7 are partially purified keratinase from isolates of *T. rubrum*

Track - 5: Protein MW Standards
Fig 17: MW of keratinase purified from *M. canis* isolates

Tracks - 1, 2, 3, 5, 6, 7 are partially purified keratinase from isolates of *M. canis*

Track - 4: Protein MW Standards

Fig 18: MW of keratinase purified from *M. gypseum* isolates

Tracks - 1, 2, 3, 5, 6, 7 are partially purified keratinase from isolates of *M. gypseum*

Track - 4: Protein MW Standards
3.4. DISCUSSION

3.4.1. CHARACTERISATION OF KERATINASE

Keratinolytic fungi produce complex of proteolytic enzymes (Asahi et al. 1985; Sanyal et al. 1985; Tsuboi et al. 1987 & 1989; Apodaca and McKerrow 1990; Grzywnowicz et al. 1989). In T. rubrum, for example, electrophoresis indicated the presence of enzymes with different molecular weights (Asahi et al. 1985; Apodaca et al. 1989a & 1989b). Proteolytic enzymes of keratinophilic fungi have a broad range of specificity, which can hydrolyze both soluble and insoluble proteins. Thus in the present study the keratinolytic enzyme was partially purified and characterized. Partially purified fractions were checked for keratinase activity species-wise. Those fractions, which showed positive keratinase activity was pooled and precipitated. The molecular weight of those proteins was determined using SDS PAGE.

This analysis showed the presence of 36.21 kDa (mean MW value of 6 isolates) enzymatically active protein secreted by T. rubrum, which proved to be keratinolytic (Fig. 2). Asahi et al (1985) isolated similar 36 kDa active protein from T. rubrum. Moallaei et al. (2006) isolated 37 kDa extracellular protease and identified as serine protease from Trichophyton species. Jousson et al. (2004a,b) also reported the isolation of 36 kDa fragment from T. rubrum.

Present study showed the purification of 33.19 kDa (mean MW value of 6 isolates) active protein fragment with keratinolytic activity from M. gypseum. Page and Stock (1974) reported the isolation of 33 kDa active fragment from M. gypseum culture.
supernatant. Raju et al. (2007) isolated 33 kDa and a 16 kDa active fragment of proteases from *M. gypseum* culture supernatant.

In the present study, *M. canis* was found to have active fragment of 33.26 kDa (mean MW value of 6 isolates) with keratinolytic activity. This was supported by Lee et al. (1987), who had isolated a 33 kDa active fragment and Descamps et al. (2002) had also isolated 31.5 kDa active fragment from *M. canis* culture filtrate. In the present study, *E. floccosum* secreted 31.41 kDa (mean MW value of 6 isolates) protein fragments with keratinolytic activity.

Over all, the present study showed that the molecular weight of the purified protein fractions with keratinolytic activity produced by different dermatophyte species taken for the present study was close to each other. These closely related keratinases with molecular weights of 36.21 kDa, 33.19 kDa, 33.26 kDa and 31.41 kDa may belong to serine proteases family. Although no biochemical assays were done to confirm that the active fragments were serine proteases, the molecular weight of the keratinases isolated were close to the findings of earlier workers, who had isolated and purified similar molecular weight active fragments belonged to serine protease family (Moehle et al. 2003; Markaryan et al. 1996; Anbu et al. 2008; Monod et al. 2005 & 2008). Most of the serine proteases are low molecular weight proteins in the range of 18.5 to 35 kDa (North 1982).
In the present study, it was found that structurally similar keratinases was found to be expressed by some or most of the dermatophytes. Such findings were supported by the work done by Takiuchi et al. (1984 & 1982) and Kunert et al. (1988 & 1989). A common class of proteases had also been reported from a wide range of organisms. Lin et al. (1992) and Tamilmani et al. (2008) had isolated active fragments of 33 kDa and 35 kDa from Bacillus licheniformis respectively. Moehle (2003) reported the purification of 33 kDa active fragment from Saccharomyces cerevisiae. Markaryan et al. (1996) reported the isolation and purification of 33 kDa active fragment from Aspergillus fumigatus.

Further, it was observed that serine proteases diffuse into the lower epidermis because of its lower molecular weight and caused inflammation. Minocha et al. (1972) demonstrated dermo-epidermal separation and spongiosis when fungal extracts were injected intra-dermally into excised human skin. In addition several earlier workers had reported the presence of serine proteases in infected tissues, which was evident for its role in pathogenicity (Becker et al. 1988; Gessner and Mortensen 1990; Reichard et al. 1990).

3.4.2. pH AND KERATINASE ACTIVITY

The present study on the effect of pH on the keratinase activity revealed that keratinase enzymes activity was high at alkaline pH. T. rubrum showed a high keratinase activity at pH-8 as reported earlier (Asahi et al. 1985; Apodaca and McKerrow 1989a & 1989b; Anbu et al. 2008).
In the present study *M. gypseum* showed a higher keratinase activity at pH 8 and the activity was extended to the maximum till pH 9. Page and Stock (1974) showed that the keratinase activity of *M. gypseum* had pH optimum of 7 to 9. In addition Raju *et al.* (2007) reported that the keratinase activity of *M. gypseum* was high at pH-8. In the present study *M. canis* showed a high keratinase activity at pH 8 as reported by earlier workers (Lee 1987; Brouta *et al.* 2001). In addition, Takiuchi *et al.* (1984 & 1982) also reported the isolation of complex proteolytic enzymes from *M. canis* with pH optima at pH 6.6, 8.0, and 9.5 to 10.0. However in the present study *E. floccosum* showed a high keratinase activity at pH 8.

### 3.4.3. TEMPERATURE AND KERATINASE ACTIVITY

The effect of temperature on the keratinase activity revealed that the enzyme activity was higher at temperature between 30°C and 40°C. The optimum temperature for the keratinase enzyme isolated from all the species (*T. rubrum, E. floccosum, M. gypseum* and *M. canis*) was 35°C. Malviya *et al.* (1992) isolated two fungal keratinase with an optimum temperature of 35°C and 40°C. Singh (1997) reported an optimum temperature of 28°C ±1°C for the isolation of keratinase from *Trichophyton* species. Ulfig (2003) isolated *M. gypseum* and *T. terrestre* at different temperature using hair baiting technique and concluded that the keratinolysis was optimum at temperature 29°C - 33°C. Raju *et al.*, (2007) also reported that the extracellular keratinase of *M. gypseum* showed an optimum activity at temperature 35°C. On the other hand several reports proved the thermostability of the keratinase enzymes isolated from dermatophytes (Ruey *et al.* 1969; Lee *et al.* 1987; Brouta *et al.* 2001). Present study support the fact that the
ability of the keratinase enzyme withstanding at higher temperature (40°C) enable them to survive in the environment by utilizing the keratinaceous substrates until the dermatophyte encounters a host of its own. Once the dermatophyte encounters the human host, the thermoflexibility of the enzyme helps the organism to withstand the human body temperature and enable to establish itself for the manifestation of infection.