Results

The erode enzyme of all the variants of *T. rubrum* showed the maximum exocellular protease activity in the Citrate-phosphate buffer pH 7.0, temperature 35°-37°C, substrate concentration of 1% and incubation time of 1 hour (Fig. 3, 4, 5, 6).

Section IV

Determination of Suitable Substrate for Extracellular Protease Production

Materials and Methods

Substrates like Gelatin, Egg albumin and Casein were used to study their effect on the extent of protease production. Each of these substrate was used separately and added in their fixed composition to the basal synthetic medium.

To 1l of the distilled water, 10g of substrate, 0.7g of KH$_2$PO$_4$, 0.3g of K$_2$HPO$_4$, 0.5g of MgSO$_4$.7H$_2$O and 1.0g of yeast extract was dissolved. Sets of different substrates were prepared for the three variants in duplicates and the media dispensed in equal amounts of 25ml each in 250ml of Erlenmeyer's flasks. The medium was autoclaved (12 lb/in$^2$ steam pressure for 30 minutes) and allowed to cool down. They were aseptically inoculated from the pregrown cultured flasks. The flasks were incubated at 30±2°C for varying number of days and their protease activity measured intermittently.

A set of each of these substrates was run under aeration to observe if there was any difference in the enzyme activity by the continuous supply of oxygen to the variants of the organism. To study the effect of substrate concentration for protease assay, substrates were used in 0.25%, 0.5%, 1%, 1.5% and 2% concentration.

Results

The cultures of the three different substrates were placed under both static condition and under aeration and the effect on the protease activity compared. For the protease assay, Casein was used in 1% concentration whereas Egg
and Gelatin were used in 0.25% concentration as the activity showed its peak in these concentrations.

The protease activity was found to be maximum in casein both in static condition and under aeration as against the two substrates. Aeration gave much better results than the static cultures. The protease activity was maximum on the 10th day (Fig. 7b).

Egg albumin too showed good protease activity but under aeration. Moderate activity was seen in its static cultures (Fig. 8).

The least activity was seen in gelatin both under static and aeration condition (Fig. 9). Since the substrate, casein, showed the maximum protease activity and a good growth, it was taken throughout investigation.

**DISCUSSION**

The dermatophytes parasitize only the superficial keratinized layers of the human body. They remain viable for long periods in such tissues detached from the body and under suitable conditions they grow and produce a variety of reproductive structures. They grow well at room temperatures in the range of 30°-35°C in the warm humid months of the year. Manipulating this character the dermatophytes including *T. rubrum* has been grown and cultured in mycological laboratories all over the world.

It is well known that morphological and cultural characters of fungi including dermatophytes were affected by different nutrient media (Benham, 1948; Georg, 1950 a,b; Razak and Rai, 1983; Rai, 1989). Of all the nutrient broths, it was observed that the growth of the variants of *T. rubrum* was very proficient with the SD broth. This medium contained peptone and glucose for the maximum growth of the variants of *T. rubrum*. Peptone is a hydrolyzed protease obtained from animals and vegetables. Hydrolytic peptone provides peptides, polypeptides, proteases, amino acids, carbohydrates, various inorganic and organic micronutrients and essential minerals for the growth of the organism. When the growth was correlated with the extracellular protease activity in the SD broth, it was observed that the enzymatic activity becomes absolutely negligible (Table-2b). This aspect of correlating nutritional pattern and enzymatic extrusion is new. However, Meevootisom and Niederpruem (1979) observed that carbon and nitrogen at a concentration of 0.5%
FIGURE 8
EFFECT OF EGG ALBUMIN ON THE EXTRACELLULAR PROTEASE ACTIVITY BY T. RUBRUM VARIANTS IN STATIONARY CONDITION

EFFECT OF EGG ALBUMIN ON THE EXTRACELLULAR PROTEASE ACTIVITY BY T. RUBRUM VARIANTS UNDER AERATION

- Avariant
- Granular
- Dysgonic
FIGURE-9
EFFECT OF GELATIN ON THE PROTEASE ACTIVITY BY T. RUBRUM VARIANTS IN STATIONARY CONDITION

EFFECT OF GELATIN ON THE PROTEASE ACTIVITY BY T. RUBRUM VARIANTS UNDER AERATION

- Avariant
- Granular
- Dysgonic
had inhibitory effect on the protease activity. Due to this reason, all nutrients, natural, semisynthetic and synthetic that contained carbohydrate and amino acid as the carbon and nitrogen sources respectively inhibited the enzyme production in the variants of the organism studied here. Sabouraud’s medium contained as much as 4% of the carbohydrate, glucose which naturally hindered the production of the enzyme. Natural media too did not have significant effect on the growth and enzyme activity of the variants, rather it showed the least activity and growth.

The malt extract contains 90-92 per cent of carbohydrate. Apart from maltose, hexose, sucrose, dextrose which comprise the carbohydrate component of malt, it has small amount of lipids, fatty and organic and inorganic acids, phosphorous, sulphur compound, and vitamins which are not easily assimilable by the strains of *T. rubrum* here. Similarly, predominant constituents of cornmeal is polysaccharides which are not also abruptly utilized by the strains of the organism. Hence, these were growth retardants as well as inhibited exocellular protease production by the strains of *T. rubrum*.

Yeast extract is prepared by autolysis or plasmolysis of cells of *Saccharomyces* and is available in powder or paste form. It is a mixture of amino acids peptides, water soluble vitamins, carbohydrates and minerals. Since the synthetic media like Czapek-Dox and Minimal broth lacked the yeast extract as a nutrient source, there was no good growth in the strains of the organism. Here in the course of further study, both carbon and nitrogen sources were replaced by casein so as to obtain the maximum enzyme production.

When basal synthetic medium with casein was used as the sole source of carbon and nitrogen and all other source of carbon and nitrogen eliminated from the medium, the growth and protease activity of the variants of *T. rubrum* got enhanced. Taking into account the basal medium, which proved to be the best nutrient source for the variants of the *T. rubrum*, optimization of growth and protease activity was done using various parameters. Most pathogenic fungi grow well at the room temperature and more vigorously on its target host’s body temperature (Lewis *et al*, 1958; Dvorak and Hubalek, 1969; McCinnis, 1980). It has been observed in the present investigation that temperature between 32°C and 35°C favored the highest growth and maximum protease production respectively. This shows that perspiration during warm humid months of the rainy season cools the superficial layers of the
human body and hence aids better growth and proliferation of the fungus. Higher
temperature beyond 45°C inhibit the enzyme production as well as the growth of the
organism.

Dermatophytes are not especially sensitive to pH and grow rather well over a broad range of hydrogen ion concentration (Howard, 1985; Ingraham et al, 1993). *T. rubrum* here, grew well over a pH range of 5-8 but the optimum growth and enzyme production was seen at pH 7.0. This establishes neutral nature for this anthropophilic fungus.

Aeration too helped in a better protease production at different days of incubation in contrast to the static cultures which suggest that these fungi are aerobic. The common pathogenic fungi require oxygen for life (Swartz, 1954; Garrison, 1961).

All the environmental parameters that induced the maximum extracellular protease production were followed throughout the present investigation in order to carry on the experiments under optimal environmental conditions.

Based on the above optimal conditions, the protease activity was seen using various protein substrates. Basal synthetic medium with gelatin, albumin and casein were taken as a sole source of carbon and nitrogen. Proteolytic activity with these protein substrates were compared both under shaking and static conditions. Casein was found to show best results for growth of all variants and protease production under both the conditions. Under aeration, however the enzyme activity increased manifold. The oxygen supply favoured the growth and protease production during the first 10 days of incubation but during later periods both the metabolic activity either remained constant or showed a slight diminishing effect.

Sanyal and Banerjee (1985) obtained pH activity curves with crude enzyme extract of *T. rubrum* which showed no decline in optimum between pH 5 and 10. Such a pH activity profile suggests the probability of existence of more than one proteolytic enzyme in the exocellular medium of this organism. This observation corroborates the findings of (Chattaway et al, 1963; Cruickshank and Trotter, 1956; Meevootisom and Niederpruem, 1975). The enzyme obtained here from the strains of *T. rubrum* showed slight variation from the above observation.

It was felt necessary to use proteins as the only sources of C and N since it was observed that easily available nutrient sources inhibited protease
production in the strains of the organism. Deprivation of such sources not only induced enzyme production but also promoted very good growth in the organism.

Using purified forms of extracellular protease, Sanyal et al (1985) determined that the enzyme activity was maximum when casein was used as the substrate during the assay. Similar observations were obtained here using three substrates as the nutrient source in the culture broth. Hence, hereafter casein was used as the nutrient source of C and N as the enzyme assay substrate.
CHAPTER-III

VARIATION OF THE NUTRIENTS OF THE BASAL SYNTHETIC MEDIUM TO EVALUATE THE GROWTH AND EXTRACELLULAR PROTEASE PRODUCTION AND PURIFICATION OF THE ENZYME

The common anthropophilic dermatophyte, *T. rubrum* that causes ringworm infection is dependent on certain susceptibility factors of the host (Padhye, 1978). The establishment of this infection is dependent not only on the aggressiveness of the pathogen or on the predisposing factors of the environment but also on certain receptiveness of the host organisms. A broad spectrum or alternatively large quantities, of a particular exoenzyme may be advantageous for enhancement of the susceptibility (Brasch and Jaldua, 1993).

Certain dermatophytes like *T. rubrum* exhibit large degree of adaptability to the parasitic habit, they can also be easily grown on a wide variety of nutrients saprophytically under laboratory conditions as pointed out by Howard (1985). The study of nutritional pattern of pathogenic fungi like *T. rubrum* serves three purposes. (1) To design media that would be selectively useful in the cultivation of pathogens from the host and from natural sources. (2) To know nutritional requirements for growth of the fungi that can be used as a means for classification of morphologically similar and identical forms. (3) To find out explanations of particular traits such as dimorphism or tissue tropism. The knowledge on these points can help understand the aggressiveness of the pathogen which is manifested clinically in the form of *tinea* (Brasch et al, 1991) due to assimilation and digestion of the host tissue by secreting enzymes.

When it is understood that micro-organism like *T. rubrum* can change their morphologic appearance and reproductive features, the workers concentrated on identifying the organism depending on its nutritional requirements (Williams, 1934; Robbins and Ma, 1945; Robbin, 1950; Hazen, 1947; Page, 1950; Georg, 1951; Georg and Camp, 1957; Johnson and Grimm, 1951; Stockdale, 1953; Stockdale, 1961; Chandra and Banerjee, 1973; Kolar and Kunert, 1977; Kunert, 1981;

Philpot (1977) introduced a simple and reliable method by which it could be shown that a particular dermatophyte could be grown on single sources of nitrogen and carbon and its ability to assimilate certain organic compounds. Later, relationships were envisaged among different species of dermatophytes on the basis of amino acid assimilation and dissimilation patterns (Singh et al, 1974; Danew et al, 1980, 1981; Kamalam and Thambiah, 1981; Thuy et al, 1981).

The differentiation of the species of *Trichophyton* may be done on the requirements of certain vitamins (Mc Ginnis & Tilton, 1994). Georg (1950) comprehensively examined the vitamin requirements of a number of species of *Trichophyton*. This work augmented the study on the various growth promoters of the dermatophytes (Georg and Camp, 1957; Rippon, 1988; Weitzman et al, 1988; Weitzman and Kane, 1991).

It is hypothesized that *T. rubrum* like all other dermatophytes, requires proteases to cleave available proteins into metabolically useable carbon, nitrogen and sulphur. This enzyme could play a prominent role not only in growth and multiplication (ensuring nutrient supply from proteinaceous components of skin, hair and nail) but also in the infection of host tissue. Minocha et al (1972) reported on the role of proteolytic enzyme on the manifestation of inflammatory reactions in hosts with dermatophyte infection. Several groups of workers demonstrated the presence of proteases and peptidases in the cells or culture filtrates of *T. rubrum* (Chattaway et al, 1963; Cruickshank and Trotter, 1956; Verma, 1966; Danew et al, 1971; Meevootisom and Niederpruem, 1979).

The diversified line of research on the *T. rubrum* and its related species primarily centered around the isolation, identification and the pure culture of the organism in the laboratory media and induction of pigmentation on it. However, isolated attempts (Meevootisom and Niederpruem, 1979; Brasch et al, 1991; Brasch and Zaldua, 1994; Apodaca & McKerrow, 1990) have been made to correlate pattern of nutrition or supply of nutrients with the exocellular proteolytic activity of
T. rubrum. In the present work, a systematic approach has been made to link the variation of any single nutrient source has any effect on the production of protease enzyme by the variants of T. rubrum. The result can help to understand the reasons why intensity of pathogenic implications of T. rubrum appear sporadically during particular seasons of the year. Deletion or avoidance of such nutrients can easily be used to exclude dermatophytic diseases.

Again, reports on the purification and characterization of the enzyme, protease, secreted from T. rubrum is scarce. The enzyme obtained here from the culture filtrates of the variants of T. rubrum isolated indigenously from human pathogenic lesions and cultured in its most suitable medium that induced growth and protease extrusion. Then it was partially purified and characterized in order to throw some light on its nature.

**SECTION-I**

**CARBON ASSIMILATION PATTERN**

**MATERIALS AND METHODS**

For the carbon assimilation study the modified basal synthetic medium was prepared with the following constituents :- Casein: 10g, KH$_2$PO$_4$: 0.7g, K$_2$HPO$_4$: 0.3g, MgSO$_4$7H$_2$O: 0.5g and yeast extract: 1.0g. These constituents were added in their fixed proportions in 500ml of distilled water. The carbon sources to be tested were added to this basal synthetic medium (BSM) in 0.5% concentration to know their effect of the growth and extracellular protease production of the variants of T. rubrum. The carbon sources tested were glucose, sucrose, lactose, starch, mannitol and glycerol. The pH of the medium was adjusted to 7.0 and the volume finally made to 11. 25ml of this medium was dispensed in 250 ml Erlenmeyer’s flasks which were autoclaved for 30 min under 12 lb/in$^2$ of steam pressure. A control for each variant was run with basal medium containing only casein as a source of carbon and nitrogen. Flasks were inoculated in the procedure described in chapter II. Dry weight of the mycelia and the protease units were calculated at every alternate days from 6$^{th}$ to 14$^{th}$ day of incubation.
RESULTS

Hexose sugar like glucose supported the best growth of the variants of the organism. But on the contrary it showed a very significant decline in the protease activity even at such a low concentration of 0.5% (Fig. 10a,b).

Diasaccharides like sucrose and lactose were least favourable carbon sources for the growth as well as protease activity. Very feeble, enzyme activity was seen when maltose was used as a carbon source. Still the extent of enzyme production and growth of the three variants was better in sucrose and lactose than that of maltose in the medium (Fig. 10a,b).

Mannitol and Glycerol were utilized well as compared to disaccharides. They promoted good growth to the variants of *T. rubrum* but could not equally support for a better protease activity. The activity was seen to decline significantly (Fig.10a,b).

Starch promoted moderate growth in all the variants of *T. rubrum* and induced a better protease activity as compared to other carbohydrates (Fig. 10a,b). A set under aeration with starch as a carbon source was taken. But when enzyme assay was done, there was no increase in the enzyme activity.

As against the carbon sources all the three variants grew well and showed the highest proteolytic activity in the control medium which had only casein as a source of carbon and nitrogen.

SECTION-II

NITROGEN ASSIMILATION STUDIES

MATERIALS AND METHODS

For the nitrogen assimilation study, basal synthetic medium was supplemented with different organic and inorganic sources individually at 0.2% concentrations. Growth and protease activity was observed at different days of incubation. The nitrogen sources tested were:— (1) Inorganic nitrogen sources: KNO₃, NaNO₃, NH₄NO₃, NH₄Cl, (NH₄)₂SO₄. (2) Organic nitrogen sources: Glycine, Alanine, Valine, Methionine, Cystine, Aspartic acid, Glutamic acid, Lysine, Leucine, Histidine, Tyrosine, Asparagine, Arginine and Glutamine. 2g of the nitrogen source to be tested was added to 500ml of basal synthetic medium, the pH of medium was
FIGURE 10a

EFFECT OF CARBON SOURCE ON THE GROWTH OF THE VARIANTS OF T. RUBRUM AT DIFFERENT DAYS OF INCUBATION IN mg

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>6th DAY</th>
<th>8th DAY</th>
<th>10th DAY</th>
<th>12th DAY</th>
<th>14th DAY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>65</td>
<td>75</td>
<td>95</td>
<td>93</td>
<td>90</td>
</tr>
<tr>
<td>Glucose</td>
<td>48</td>
<td>64</td>
<td>88</td>
<td>80</td>
<td>82</td>
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<tr>
<td>Sucrose</td>
<td>52</td>
<td>63</td>
<td>86</td>
<td>94</td>
<td>92</td>
</tr>
<tr>
<td>Lactose</td>
<td>65</td>
<td>70</td>
<td>104</td>
<td>93</td>
<td>82</td>
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<td>52</td>
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</tr>
<tr>
<td>Mannitol</td>
<td>15</td>
<td>50</td>
<td>47</td>
<td>32</td>
<td>52</td>
</tr>
<tr>
<td>Glycerol</td>
<td>20</td>
<td>49</td>
<td>49</td>
<td>36</td>
<td>80</td>
</tr>
</tbody>
</table>

Variants:
- A variant
- Granular
- Dysgonic
EFFECT OF CARBON SOURCE ON THE PROTEASE ACTIVITY OF T. RUBRUM VARIANTS AT DIFFERENT DAYS OF INCUBATION IN (UNITS) PER mg DRY WEIGHT OF THE MYCELIA

Control Glucose Sucrose Lactose Maltose Starch Mannitol Glycerol

□ Avariant ■ Granular □ Dysgonic

FIGURE 10b
adjusted to 7.0 and the volume was made up to 11 by distilled water. One test set for each variant was run with only casein as a source of carbon and nitrogen. In nitrogen assimilation studies, the same general procedure was followed as mentioned in carbon assimilation studies for determination of growth and extracellular protease by the strains of the organism.

RESULTS

In comparison to the control sets taken, both the inorganic and organic nitrogen compounds did not help the organism in their growth and protease production excepting a few variants (Fig. 11a,b; 12a,b).

In the inorganic nitrogen compounds best growth was seen when the basal media was supplemented with NaNO₃. KNO₃ too promoted good growth in all the three strains. Moderate growth was seen in NH₄NO₃, NH₄Cl and (NH₄)₂SO₄ (Fig. 11a).

The protease activity was best seen in KNO₃ as compared to other inorganic forms in all the 3 strains in the 10th day of incubation. NH₄Cl also supported a good protease activity in all the cases on the same day. (NH₄)₂SO₄, NH₄NO₃ and NaNO₃ did not have any inducing effect on the activity (Fig. 11b).

Among the organic nitrogen compounds, Asparagine favoured the best growth of all the three variants of T. rubrum. Lysine, valine, asparatic acid, glutamic acid and glutamine supported good growth of the organism. Glycine, alanine, methionine, histidine and tyrosine favoured moderate growth (Fig. 12a). No growth was seen with cystine. In the medium decline in protease activity was seen in all the amino acids tested as against the control media which showed that these amino acids had no inducing effect on the protease production (Fig. 12b). However decline was not much significant in histidine and methionine whereas a remarkable retardation in enzyme activity was seen in valine, glycine, glutamic acid and aspartic acid (Fig. 12b).
FIGURE 11a
EFFECT OF INORGANIC NITROGEN SOURCE ON THE GROWTH OF THE VARIANTS OF T. RUBRUM AT DIFFERENT DAYS OF INCUBATION IN mg

6th DAY

8th DAY

10th DAY

12th DAY

14th DAY

Control (NH₄)₂SO₄, NH₄NO₃, KN₀₃, NaNO₃, NH₄Cl

Variant Si Granular □ Dysgonic
FIGURE 11b

EFFECT OF INORGANIC NITROGEN SOURCE ON THE PROTEASE ACTIVITY OF T. RUBRUM VARIANTS AT DIFFERENT DAYS OF INCUBATION IN (UNITS) PER mg DRY WEIGHT OF THE MYCELIA

Control (NH₄SO₄, NH₄NO₃, KNO₃, NaN₂SO₄, NH₄Cl)

Variants

Granular

Dysgonic

6th DAY

8th DAY

10th DAY

12th DAY

14th DAY

Control

(NH₄)₂SO₄

NH₄NO₃

KNO₃

NaN₂SO₄

NH₄Cl

Avariant

Granular

Dysgonic