CHAPTER-I
IDENTIFICATION OF THE HUMAN PATHOGENIC 
DERMATOPHYTIC STRAINS AND SCREENING OF THE 
EXTRACELLULAR ENZYMES

Introduction

*Trichophyton*, *Microsporum* and *Epidermophyton* are the dermatophytes that generally attack the superficial layers of skin and cause acute chronic infections. These superficial and cutaneous mycoses are the most prevalent in India and are responsible for significant outdoor patient attendance in many leading hospitals of India (Murdia, 1987; Knudson, 1987). The fungal infections are common in the regions where warm humid climate of rainy season prevails.

Reports of the occurrence of the disease from several parts of India including Orissa are available in the literature (Panda et al, 1967; Kurup and Ananthnarayan, 1969; Verma et al, 1970; Stephen and Rao, 1975; Mehta et al, 1977; Sharma et al, 1980; Shukla et al, 1983; Murdia, 1987). However the status of disease at Rourkela, a thickly populated town of Orissa, has not been systematically studied yet due to many reasons. Lack of awareness about the disease, its appearance in sporadic form all round the year and improper methods of identification of the cause of disease can be cited as the reasons.

Enzymes play a prominent role in the pathogenicity of the dermatophytes (Rippon, 1968; 1969; Das et al, 1977; Hellgren, 1980) which may be harmful for the host tissues when liberated by the fungi. The formation and release of extracellular enzymes by the fungi during sporulation or growth is of common occurrence (Howard, 1985).

With the above points in sight, the dermatophytic incitants at Rourkela were isolated and identified following the standard methods. The soil from the garbage/dumping yards containing the keratin at the patients' locality were collected to ascertain the source of primary infection of the pathogens. The variants of the isolated organism were grown in certain standard enzyme assay media to determine the enzymes extruded from it. The identification of enzymes can help to
ascribe the pathogenic characteristics of the organism.

SECTION I

ISOLATION AND IDENTIFICATION OF THE ORGANISM

(a) ISOLATION OF THE ORGANISM:

The samples were collected for a period of one year from the suspected patients having cutaneous skin infections who visited the Skin and Veneral disease department of Ispat General Hospital, Rourkela, Orissa, India.

The scrapings were taken aseptically on sterile filter papers after applying 70% alcohol on the affected areas as per the method of Kite (1982). A part of scrapings was mounted on 10% KOH and observed directly under the microscope to detect the occurrence of fungal elements (spores and hyphae). Remaining part of the material was inoculated to SDA medium (Dextrose: 40g, Peptone: 10g, Agar: 15g) dissolved in distilled water and volume was made upto 1l at pH 5.6 containing 0.5 g/l of cycloheximide and 0.05 g/l chloramphenicol and the incubation was done at 30±2°C. All the cultures were held for three weeks for the mycotic growth and the negative ones were discarded. A dermatophyte test medium (Distilled water: 1l, Phytone: 10g, Dextrose: 10g, Agar: 20g, Phenol red solution: 40ml, 0.8N Hcl: 6ml, Actidione: 0.5g, Gentamycin sulphate: 0.1g of active drug=100µg/ml, Chlorotetracycline Hcl: 0.1g=100µg/ml) was prepared for the confirmation of dermatophytes (Rebel and Taplin, 1979).

Samples from the patients' house hold garbage sites and sewage systems of their locality were collected. The dermatophytes, if any, in the samples were isolated by the soil dilution method and hair baiting method as described below.

(i) Soil dilution method:

Soil which was collected from the garbage sites of the patients' locality was air-dried, powdered and passed through a 2mm sieve before use, 10g of the soil was dissolved in 100ml of sterile distilled water and kept for 2 hours in shaken condition.

Sabouraud’s medium containing cycloheximide and chloramphenicol was plated in petridishes for 72 hours. Serial dilution (10⁻³ and 10⁻⁴)
of soil samples was made in sterile distilled water and 0.5ml portions were inoculated into the petridishes containing the sterile SDA medium in the chamber and incubated at 30±2°C for 1 to 2 weeks.

(ii) Hair bait method:

The petridishes were half filled with garden soil and sterilized at 20lb/in² steam pressure for 30 minutes for 2 days. Then the soil was moistened with sterilized pond water (Not to be water logged). Bits of child hair were sprinkled over the petridish with soil. The floor sweeps were also sprinkled over the hair and incubated at room temperature for one week. After the desired incubation period the hair bits were searched for any growth of the fungus.

(b) IDENTIFICATION OF THE ORGANISM :

After the incubation for a week, the pathogenic fungi started growing on the test cultures. They were removed for macro and micromorphology identification. On being observed under microscope after staining with lactophenol cotton blue, the colony morphology and the mycelial structure of the organisms with their conidia were observed.

The samples were detected to be variants of *T. rubrum*. Tests like potato carrot agar medium, pigmentation on PDA medium, urease test, thiamine and histidine test of nutrition and the standard in vitro hair perforation tests were also done. Moreover, the identification of fungal organisms was further confirmed in the Mycology division of the School of Tropical Medicine, Calcutta, West Bengal, India.

(c) (i) Sporulation in potato-carrot agar :

Potato-carrot agar was used to stimulate sporulation of *T. rubrum*. This medium was prepared by combining 20g of potatoes with 20g of carrots, each previously peeled and cut into small pieces. These were boiled in 11 of distilled water for 30 minutes. After steaming, 20g of agar was added and the medium was autoclaved at 12lb/in² steam pressure for 15 minutes, dispensed into tubes in 15 ml portions and sloped. This agar was inoculated with pinpoint inocula of the test fungus and incubated at room temperature for 28 days. After 7, 14 and 21 days a small amount of growth was aseptically removed from the tubes and mounted into a drop of
lactophenol cotton blue for microscopic examination. The detection of typical *T. rubrum* microspores or macrospores or both was considered to be positive result (Rebel and Taplin, 1970). The presence of typical, *T. mentagrophytes* microspores or macrospores or both, or complete absence of spores was recorded as negative results, indicating no occurrence of *T. rubrum*.

**(ii) Pigment production on potato dextrose agar:**

Potato dextrose agar was prepared (peeled potatoes 250g in 100 ml distilled water boiled and filtered, then 20g of dextrose and 20g of agar was added to the filtrate and the volume was made to 1l by distilled water), aseptically dispensed in 15 ml portions into tubes, sterilized at 15 lb/in² steam pressure for 10 min. and slanted. The medium was inoculated with a pin point inoculum of test fungus and incubated at room temperature for 28 days. The cultures were examined for the pigment production at 7 days intervals.

**(iii) Urease test medium :**

To 1l of distilled water, Peptone: 1g, NaCl: 5g, KH₂PO₄: 2g, Glucose: 5g, and Agar: 20g were added. 6ml of phenol red was added and the medium was sterilized by autoclaving at 10 lb/ in² pressure for half an hour. It was then cooled to 50°C and 100ml of sterilized urea (20%) was added and the medium was dispensed into plates. The fungus was inoculated in the culture chamber.

**(iv) Hair perforation test:**

This test was based on the capability of *T. mentagrophytes* to perforate hair and the inability of *T. rubrum* to do so. About 1cm. strands of pre-pubertal human hair were placed in the petridishes and sterilized by autoclaving (10lb/in²) steam pressure for 10 min. 25ml of distilled water and 2-3 drops of sterile, 10% yeast extract was added. Fungi were inoculated into it. Perforations were examined at intervals of 1 week upto 4 weeks by removing singly hair from the plates and scanning them under the microscope.

**(v) Media to test independence of the variants to thiamine and histidine:**

Glucose: 40g, MgSO₄: 0.1g, KH₂PO₄: 1.8g, Histidine solution: 25ml (150mg of the histidine dissolved in 100ml of distilled water), Agar: 20g were taken and the volume was made to 1l by distilled water and autoclaved at 12 lb/in².
steam pressure for 15 minutes. Control histidine was also prepared side by side. Same procedure was followed for thiamine test by substituting thiamine solution by histidine solution. Thiamine solution was prepared by adding 10mg of thiamine HCl with 11 of distilled water (pH 4-5).

**CULTURE METHODS:**

The variants of the organism were grown in Sabouraud’s dextrose agar media at 30±2°C for two weeks and then preserved at 40°C. Subculturing were done by transferring it into fresh media at every two week intervals. After every subculturing growth of the variants was observed under the microscope so as to avoid contamination of any other organism.

**TRANSFER OF INOCULUM:**

The required medium was dispensed into the Erlenmeyer’s flasks and autoclaved for 30 minutes at 12 lb/in² of steam pressure. The flasks were closely tightened with cotton plugs and covered with aluminium foils. Pregrown inoculum from the 3-4 days old basal medium was transferred aseptically into the flasks inside a sterilized chamber. Inoculated flasks were left undisturbed for 12 hours and then transferred to the incubator at 30°C. The incubation time was counted from the time, the flasks were kept in incubator.

**GROWTH OF THE CULTURED FUNGI:**

Growth of the fungi was recorded at 4th, 6th, 8th, 10th, 12th and 14th day of incubation. Dry weight of the mycelia was considered as the index of growth. The flasks were removed in triplicates for the purpose. Mycelia of the individual flasks were harvested in Whatman 11cm No.1 filter paper and were washed with distilled water to free it of culture filtrate. The filter papers used were dried at 85-90°C for 2 days and weighed. The dry weight of the mycelia were determined by comparing the difference in the initial and final weights. The culture supernatant (filtrate) was used as a source of the crude enzymes. The supernatants were preserved at the freezing temperature for extracellular protease analysis.
RESULTS

A total of 96 out of 250 suspected cases (38.4%) were observed to be culture positive for dermatophytes. The commonest clinical type was found in following order: tinea cruris, tinea pedis, tinea mannum and tinea unguium. The etiologic agent identified from among all the clinical lesions was T. rubrum. Patients in the age of 21-30 years were vulnerable to the infection and males outnumbered the females, when the sex of the patients was taken into the account in the preliminary analysis.

Macro and micromorphological studies revealed that 3 variants of T. rubrum were responsible for the disease. The variants were dysgonic, granular, and a new variant (which was classified as a variant type) and their number were 50, 45 and 1 respectively. All the variants were independent of thiamine and histidine. The variants showed a good tendency in variation of their characters. With a slight change in their environment they underwent irreversible changes purely for the sake of convenience. The features they exhibited are detailed below.

(i) GRANULAR TYPE WITH MACROCONIDIA (A VARIANT TYPE):

Its morphology is very similar to granular type. Cylindrical macroconidia with 8 or 9 cells or in some cases 6 to 9 cells tend to fragment into 2-3 cells length. Clusters of round to grape shaped microconidia are seen. Urease activity is same as granular type. Rate of growth has been observed to be slower in subcultures than primary cultures in all the variants. The cultures of the isolated forms were maintained in Sabouraud's dextrose agar medium, since it contained easily utilizable forms of carbon, nitrogen and vitamin sources. The pH of the medium was adjusted to 5.6 and the cultures were transferred in every 15 days and regrown to facilitate multiplication of the species and avoid any chances of contamination (Plate 1a,b,c).

(ii) GRANULAR TYPE:

This form is powdery, glabrous and heaped up with radial furrows. Greenish pigment is seen in 3 weeks old culture. Many chlamydospores are seen in old cultures. Variable number of round to clavate microconidia are seen. Absence of macroconidia in PDA medium, growth is slow but zonal clearing is seen.
a: Side view of 4 week old culture of the variant type similar to granular type with radial furrows.
b: Reverse side of the colony with greenish pigmentation which later became red pigmented.
c: Smear from the culture showing hyphae with numerous microaleuriospores and one macroaleuriospore.
a: 4 week old culture of granular type showing powdery velvet colour

b: Reverse side of the colony showing deep red pigmentation

c: Smear from the culture showing variable number of round to clavate microaleuriospores with chlamydospores
a: 4 week old culture showing the front view of dysgonic type

b: Reverse side showing wine red pigmentation

c: Smear from the old culture of dysgonic type showing branched stunted hyphae with pyriform microaleuriospores with some chlamydospores
(iii) DYSGONIC TYPE:

These forms are very fast growing, fluffy, woolly and suede like and changes to granular forms in 4 weeks old culture. Feathery peripheral growth are well marked. Chlamydomspores with varying number of only pyriform microconidia are seen. After 2-3 times of subculturing greenish-yellow pigment is developed on the reverse side of one week old culture tubes. After 2-3 weeks red pigment is seen. This type showed a very weak urease activity (Plate - 3a,b,c).

SECTION II

SCREENING OF DIFFERENT EXTRACELLULAR ENZYMES

Enzymes secreted by the three variants of \textit{T. rubrum} were either screened qualitatively or quantitatively following the assay method of (Hankin and Anagnostakis, 1975).

QUALITATIVE ASSAY:

LIPASE: The medium described by Sierra in 1957 was used to detect the production of lipase enzyme. The medium for lipase contained Peptone: 10g, NaCl: 5g, CaCl$_2$.2H$_2$O: 0.1g, Agar: 20g, per 1l distilled water at pH 6.0. Tween 20 was sterilized separately by autoclaving for 15 minutes at 15lb/in$^2$ pressure. 1ml of it was added per 100ml of sterile basal medium. Formation of lipolytic enzyme by the colony was seen as either a visible precipitate due to the formation of crystals of calcium salt of lauric acid and liberated by the enzyme or as a clearing of such a precipitate around a colony due to degradation of fatty acid.

AMYLASE: Ability of fungi to degrade starch was used for determination of ability to produce amylolytic enzyme. The medium used contained Nutrient agar: 20g, 0.2% soluble starch per 1l at pH 6.0. After 3-5 days of incubation the plates were flooded with Iodine solution and the yellow zone around the colony in a blue medium indicated amylolytic activity.

UREASE: Presence of urease was detected by modified Christensen's medium. It contained Peptone:1g, NaCl:5g, KH$_2$PO$_4$:2g, Glucose:5g, Agar:20g to 1l of distilled
water. 6ml of phenol red (0.2% in 50% alcohol) was added and the medium was sterilized by autoclaving at 10 lb/in² of steam pressure for half an hour. Then it was cooled to 50°C and 100ml of sterilized urea was added immediately and dispensed into the plates.

**PROTEASE:** The medium that contained gelatin as a protein source was used to detect the production of proteolytic enzymes. The medium contained nutrient agar with 0.4% gelatin, pH 6.0. 0.8% of gelatin in water was sterilized separately and added to the nutrient agar at a rate of 5ml per 100ml medium. Protease activity was observed by degrading and clearing opaque gelatin around the colonies grown in solid media flooded by saturated solution of ammonium sulphate.

**KERATINASE:** Keratinolytic activity was known by the following qualitative test with liquid broth. The keratin substrates used were human hair and nails. Substrates were washed several times in distilled water and air-dried and then were sterilized by ‘dry autoclaving’. Medium contained Glucose: 1g, KNO₃: 5.4g, K₂HPO₄: 10g, MgSO₄·7H₂O: 0.5g, Thiamine: 100mg and Yeast extract: 2mg. The pH of the medium was adjusted to 7.0 before sterilization.

An aliquot of 30ml of the above medium was taken in 250ml of Erlenmeyer flask and sterilized at 15 lb/in² steam pressure for 15 min. To each flask, 250 mg of sterilized keratin was added. The flasks were inoculated with an inoculum disc of 6 mm diameter obtained from 8 day old colony of the fungi.

Three sets of the flasks were run as follows.

**Fungus Control:** Containing 30 ml of the basal medium and a disc of fungal inoculum.

**Keratin Control:** Containing 30 ml of the basal medium and 250 mg of keratin substrate.

**Test Samples:** Containing 30 ml of the basal medium and 250 mg of keratin substrate and a disc of fungal inoculum. Flasks were incubated in duplicates as static cultures for 30 days at 28°C.

Degradation of keratin was monitored by determination of percentage weight loss of each of keratin substrates using the weight loss method (Fergus, 1969; Jain and Agarwal, 1977; Geetha Singh, 1981; Chandrakeet Singh, 1993; Derich Vijay Singh, 1993). After 30 days, the flasks were harvested and the mycelial mats along with keratin substrates were removed and dried at 80°C for 24 hrs. The total dry weight in
each case was determined and compared with the weight of substrate control. If the substrate with yield mycelia weighed more than the weight of control, fungus was considered to be non-keratinolytic and increased weight attributed to the mycelial production due to utilization of nutrients from basal medium and substrate. It was recorded as zero loss of weight. If the fungus with substrate weight less than control, the fungus was considered to be having keratinase activity. Net loss of the substrate was attributed to the loss of non-recoverable production of keratin degradation.

**QUANTITATIVE TEST:**

For quantitative test the variants of *T. rubrum* were grown in Sabouraud's dextrose broth in 250 ml Erlenmeyer flasks at 30°C±2°C. After 10 days of incubation, the mycelia were separated from the medium by filtration. Filtrate was centrifuged at 10,000 rpm for 20 minutes in cold centrifuge to get rid of mycelial debris. The supernatant was stored at 5°C to be used for enzyme assays. For the assay of L-amylase, lipase and protease, medium composition was changed slightly. Since free glucose in the medium inhibited the enzyme synthesis (Clementi *et al.*, 1980), dextrose in SD broth was replaced by starch to avoid the interference of glucose in the assay system.

Besides, the SD medium, a set of certain inducers were taken for the assay of lipolytic activity. Medium composition as glucose: 1%, K$_2$HPO$_4$: 0.5%, KNO$_3$: 1%, MgSO$_4$: 7H$_2$O: 0.25%, Corn steep liquor: 1% and rape seed oil: 2%, pH being adjusted to 6.5. Protease being an inducible enzyme requires the presence of its substrate. It was observed that presence of glucose or amino acids source reduced the protease production (Meevootisom and Niederpruem, 1979).

Hence, the synthetic medium with the following constitution was used: Casein powder: 10g, KH$_2$PO$_4$: 0.7g, K$_2$HPO$_4$: 0.3g, MgSO$_4$.7H$_2$O: 0.5g and yeast extract:1g in 1l of distilled water, pH being adjusted to 7.0. Assays of enzymes were done by following procedure.

**L-AMYLASE:**

Amylolytic activity was determined by the method of Noelting and Bernfeld (1948). Assay system consisted of 2ml of culture filtrate, 0.5ml of 1% starch solution and 0.3ml of sodium phosphate buffer, pH adjusted to 7.0 and incubated at 37°C for 15 minutes in a water bath. The reaction was stopped by adding
1ml of 3,5 dinitrosalicylic acid and subsequently heated to develop colour as described by Sumner (1924) and read using Erma colorimeter using green filter.

**LIPASE:**

Lipase was assayed after titrating it with standard alkali, the fatty acids, liberated from lipid substances by the action of the enzyme as described by Bier (1965). Olive oil was used as the substrate. Emulsification of substrate and assay of the enzyme were made in the following manner- 2.7g of gum arabic was dissolved in 5g of oil and the volume was made to 100 ml. A clear solution was prepared by using a mixer grinder and finally with an ultra sonicator, pH adjusted to 7.0.

To 12 ml of the substrate, 2.5 ml of the enzyme were added and incubated at 30°C for 5 hours. Reaction was terminated by 2 volume of acetone : ethanol mixture (1:1 v/v). The liberated fatty acids were titrated with the indicator. For reaction blank, acetone-ethanol were added, before the enzyme source and rest of the procedure was the same. 1 ml of N/50 NaOH solution considered as 10 lipase units.

**L-ASPARAGINASE:**

Asparaginase activity was obtained by estimating the amount of ammonia formed by the enzymatic activity. The substrate L-asparagine dissolved in 0.1(M) phosphate buffer, pH 7.0 was diluted to make a final concentration of 0.01(M). A reaction mixture containing 0.3 ml of 0.01(M) L-asparagine, 1.4 ml phosphate buffer, pH 7.0, 0.3 ml of enzyme prepared to make total volume of 2 ml was incubated at 30°C for 1 hour.

In all experiments 2 control series were maintained besides the reaction series. Series 1: Reaction mixture, Series 2: Enzyme blank, Series 3: Substrate blank. Reaction was stopped with 0.5 ml of 20% TCA and mixture centrifuged at 10,000 rpm for 10 minutes. Clear supernatant was used for ammonia assimilation. L-asparaginase activity was expressed as µg of ammonia liberated per ml of culture filtrate in 1 hour.

**UREASE:**

Urease activity was assayed by a modified method of Sumner and Hand (1928). Reaction mixture consisted of 0.25 ml of 3% urea, 0.5 ml of 0.1(M) phosphate buffer pH 7.0 and 0.25 ml of enzyme extract. The mixture was incubated at 30°C for 1 hr. The reaction was terminated by ZnSO₄-NaOH and ammonia liberated
was estimated by indophenol reaction (Kaplan 1969). The colour developed was read in Erma make Colorimeter using red filter (660nm). The control was run parallely and the activity if any was expressed in terms of μg of ammonia liberated per ml of enzyme per hour obtained referring to a standard graph prepared with known concentrations of (NH₄)₂ SO₄.

**PROTEASE:**

Protease assay was performed according to the method of Anson (1938) with a few modifications. To 1.0 ml of 1% (w/v) substrate solution (casein) in 0.1M Citrate-phosphate buffer (pH 7), 0.2ml of enzyme solution (culture supernatant) was added. The reaction mixture was kept at 30°C for 1hr after which the reaction was stopped by the addition of 20% TCA. After a lapse of 10 minutes the reaction mixture was centrifuged at 10,000 rpm for 10 minutes in order to remove the unhydrated proteins. To 1 ml of the supernatant, 5 ml of 0.275(M) sodium carbonate, 2 ml of distilled water and 0.5 ml of 1:1 diluted folin's phenol reagent were added. The intensity of the blue colour developed was measured in Erma make colorimeter using red filter (660nm). All conditions remaining the same a control was run in which TCA was added before the addition of enzyme. 1 unit of proteolytic activity was considered as the amount of enzyme which liberates 1 μmol equivalent of tyrosine under experimental conditions.

**KERATINASE:**

The activity was assayed by modification of Yu et al (1968) method. The substrate was white guinea pig hair, extracted with chloroform method and was dried. The unsterilized hair (50 mg, 1-3 mm long) was suspended in 5.4 ml of 0.02(M) phosphate buffer containing 1g MgSO₄ (pH 8.0) and 0.6 ml of keratinase solution. It was incubated at 37°C with gentle shaking for 2 hrs. The control samples were made in the same way except that keratinase solution had been kept at 100°C for 10 minutes.

The incubated solution was immersed in ice water bath for 10 minutes to stop the reaction. Hair was removed by filtration with filter paper and the absorbence of filtrates were measured at 280nm using a spectrophotometer. 1 unit of keratinous activity was corresponded to an increase in absorbency value by 0.1.
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RESULTS

In the qualitative and quantitative tests, it was determined that the variants of *T. rubrum* liberated enzymes like protease and lipase very well. Positive urease activity was obtained in case of granular and avariant type but a weak urease activity was found in dysgonic type (Table-1).

No L-amylase and L-asparaginase activity and a very feeble keratinolytic activity were obtained in all strains of *T. rubrum*. Keratinase activity was not found when human hair (adult) was supplied as a substrate. As positive results were not obtained with these enzymes hence they were abandoned in the course of further analysis (Table-1).

DISCUSSION

It was observed during one year of survey that the three variants of *T. rubrum* were the only cause of dermatophytic infections at Rourkela (Plate 1,2,3). This finding as to the predominance of only *T. rubrum* among anthropophilic dermatophytic incitants has been corroborated by many workers all over the world. Sinski and Lee (1991) observed that 54.8% of ringworm infections are caused by *T. rubrum*. It causes the disease called onychomycosis. Kam et al, 1997 in Honkong, Santios et al, 1997 in Brazil observed that *T. rubrum* causes 58.6% of
<table>
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<th>Medium</th>
<th>Enzyme</th>
<th>Substrate</th>
<th>Variants of <em>T. rubrum</em></th>
<th>Granular</th>
<th>Dysgonic</th>
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<tr>
<td>Starch 4% 1% peptone</td>
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<tr>
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<td>Asparagine (0.01M)</td>
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<tr>
<td>Inducer containing Modified Sabouraud's Broth</td>
<td>Lipase</td>
<td>Emulsified Olive Oil (5g/100ml)</td>
<td>++</td>
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<td>++</td>
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<tr>
<td>Modified Sabouraud's Glucose Peptone</td>
<td>Urease</td>
<td>Urea (3%) (µg ammonia/ml/hr)</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Synthetic medium</td>
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<td>Casein (1% and 5%)</td>
<td>++</td>
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<tr>
<td>Synthetic medium</td>
<td>Keratinase</td>
<td>Hair (250 mg/ml)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Synthetic medium</td>
<td>Keratinase</td>
<td>Nail (250 mg/ml)</td>
<td>feeble</td>
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“++” : Very good active; “+” : positive activity; “-” : no activity.
dermatophytosis followed by *T. mentagrophytes* (25.3%). Epidemiologic survey of the superficial fungal diseases performed outside United States demonstrated that most cases of onychomycosis (Kenna and Elewski, 1996) and *tinea pedis* are caused by *T. rubrum*. In *tinea* and onychomycosis this dermatophyte appeared in approximately 95% and 82% isolates respectively.

Since last 3-4 decades in India, the reports published so far unequivocally, put *T. rubrum* as the most common dermatophyte isolated from various lesions followed by other dermatophyte species of *Trichophyton, Microsporum* and *Epidermophyton* (Kalra et al, 1964; Panda et al, 1967; Prasad and Prakash, 1979; Sharma et al, 1980; Murdia, 1987; Williamson and Lyer, 1992; Chakraborti et al, 1992).

However, isolation and identification of the variants of *T. rubrum* only here at Rourkela is unique. The organism might have evolved to the present from at Rourkela, India, due to wide ranging changes in the ecology, environment, life style patterns of the denizens of this city. The dramatic changes are attributed to the establishment of a giant public sector steel plant in the late fifties of the last century and development of many ancillary industries.

As detailed in the introductory chapter, a single species of dermatophyte may cause a variety of clinical manifestations in different parts of human body which are clinically called "*tinea". Systematic ringworm infections of body clinically called *tinea corporis* (20.4%) was predominating one followed by *tinea cruris* (13.6%) and *tinea pedis* (9.2%). High incidence of *tinea corporis* was noted in many parts of India like in Vishakapatnam (Rao et al, 1970); Ahmedabad (Shah et al, 1975); Manipal (Stephen et al, 1975); Kunool (Naidu et al, 1976) and Chennai (Sentamilselvi et al, 1997-1998). It was observed that the maximum manifestation of the disease becomes during the months of September-October at Rourkela. The warm, humid climate, temperature varying between 30°-37°C helped the growth and sporulation of the fungal organism and the spread of the disease. The organism remained inactive during most parts of the year in human hosts but with the onset of favourable condition, they become active in form of pathogenic lesions.

It was observed earlier (Das, 1996) that out of the three variants isolated here, dysgonic type was a slow growing one. In the present investigation, however, it was observed that the variant has changed its habit and was growing very...
fast in the culture medium. It may be due to continuous subculturing of the organism in the artificial media and its adaptation to grow well under that condition. The other two variants have been observed to retain their habit. Out of the three variants dysgonic and granular forms have been reported else where (Jawez et al, 1978; Khare, 1983; Shukla et al, 1984) but the identification of the new ‘variant’ form was characteristically different.

The cultures obtained from the skin scrapings of the patients, who visited Ispat General Hospital, were maintained in the Sabouraud’s Dextrose Agar medium which promoted the maximum growth of the organism.

Dasgupta and Shome (1960) have, in a qualitative and quantitative study of extracellular enzymes of *T. rubrum* and *T. mentagrophytes* demonstrated L-amylase produced by them. Several workers (Macfadyan, 1895; Roberts, 1899; Goddard, 1934; Choudhury, 1985) failed to detect extracellular amylase activity in certain *T. rubrum* strains. This may be due to the functional differences of the organism although they exhibit similarity in morphological features.

None of the variants showed extracellular L-asparaginase activity. Shome and Verma (1964) observed a moderate amino acid oxidase activity in *T. mentagrophytes* and *T. rubrum*. Bentley (1953) demonstrated strong asparaginase activity in *M. canis* and *M. gypseum* and weak activity in *T. mentagrophytes*.

On the screening of keratinolytic activity, it was observed here that the variants produced keratinase feebly when human nail was used as keratinous substrate. However the activity was not observed at all when human hair was used as the substrate. It was shown that the fungus infected thickly keratinized skin and nail but not the hair. Keratinolytic activities of dermatophytes like *T. rubrum*, *T. mentagrophytes* and *M. gypseum* were already been described (Danew et al, 1974; Danew et al, 1980; Hiao et al, 1982). Further keratinases have been identified and purified in crude culture filtrate of *T. rubrum* or related *Trichophyton* species (Rippon and Varadi, 1968; Yu et al, 1971; Tsuboi et al, 1989, 1992; Tanaka et al, 1992; Lambkin and Hay, 1994) and in other dermatophytic and keratinophilic fungi (Kushwaha, 1983; Chandrakeet Singh, 1993; Derick Vijay Singh, 1993). *T. schoenleinii* found that human (adult) hair and human nail did not have such a single activity with keratinase. Rosselet and Frenk (1990) observed that *T. rubrum* most frequently affecting nails in vivo, had low aggressiveness in the in vitro
condition, when nail was used as the substrate.

The possession of extracellular urease may have been advantageous for a dermatophytic species in different ways in addition to the fact that urea has been demonstrated to be a good source of nitrogen for dermatophytes (Hejtmanek, 1960; Zeigler, 1961; Mathison, 1962; Sanyal and Banerjee, 1983). With the help of this enzyme, it may be able to utilize urea secreted through sweat (Sanyal and Banerjee, 1983). Dasgupta and Shome (1960) reported extracellular presence and intracellular absence of urease in *T. rubrum*. Sen (1973), however found the occurrence of intracellular urease in *T. rubrum* and *T. mentagrophytes*.

Das and Banerjee (1977, 1982) studied the lipolytic activity in Sabouraud's dextrose medium in a strain of *T. rubrum*. Nobre and Viegas (1972) reported that the enzyme production gets reduced after subculturing. Deshmukh and Agarwal (1982) confirmed that the lipase was synthesized by *T. rubrum* with protease and phospholipase and suggested the enzyme producing capability of the fungus.

There are several reports on protease activity and on the varied aspects of enzyme of *Trichophyton* species (Mikhail, 1970; Takuichi et al, 1983; Ashahi et al, 1985). Sanyal et al, (1985) studied the aspect of this enzyme and their results corroborates the present findings.

Although more than one enzyme have been detected to be excreted to the medium by the variants of the organism, the enzyme, protease, was only taken in succeeding chapters for a detailed study. Protein, being a very important constituent of the skin and being the building block molecule of the cell, which is the ultimate unit of life, it was felt necessary to study the enzyme which hydrolyses such macromolecules. Moreover, the enzyme has a unique place in the living systems since it performs many degradative and assimilative functions in the living beings.

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