CHAPTER —II

ASSAY OF THE EXTRACELLULAR PROTEASE

_Candida albicans_, an opportunistic fungus is responsible for increasing morbidity and mortality in the immuno-compromised hosts (Reingold _et al_, 1986; Richardson, 1991; Morrison _et al_, 1993). The basic characteristics associated with the pathogenicity of this normally benign, endogenous commensal include predisposing host factors (Morrison _et al_, 1986; Odds, 1988), conversion from yeast to pseudohyphal form, (Barnes _et al_, 1983; Anderson and Odds, 1985) increased adherence to epithelial or mucous membranes (McCourtie and Douglas, 1984; Ghannoum and Elteen, 1986) and the production of an extra–cellular proteinase (Staib, 1969; MacDonalds and Odds, 1980; Kwon-chung _et al_, 1985; Morrison _et al_, 1993).

A potential virulence factor of _C.albicans_, that was first identified by Staib (Staib, 1965) and described by Remold _et al_ (1968) has been studied by a number of laboratories (Rüchel, 1981; Borg and Rüchel, 1988) over the last twenty–five years is the secreted acid proteinase (White and Agabian, 1995). Among a number of hydrolytic enzymes, this enzyme, protease (proteinase), has been given a great deal of importance in causing disease by a number of workers (Remold _et al_, 1968; Germaine _et al_, 1978; Rüchel, 1981; MacDonald and Odds, 1983; Odds, 1985; Rüchel _et al_, 1985; Kwon-chung _et al_, 1985; Ghannoum and Elteen, 1986; Borg and Rüchel, 1988; Banerjee _et al_, 1991). Such an activity could play an important role in facilitating adhesion of cell or in promoting tissue penetration.

Mechanism of _Candida_ invasion of skin are not known, although mechanical and enzymatic events have been hypothesized (Howlett and Squeir, 1980; Borg and Rüchel, 1988; Ray and Payne, 1988; Ray and Payne, 1990). By inhibition studies, it has been shown that the enzyme plays a crucial role in fungal adherence and in invasion of the epithelium (Borg and Rüchel, 1988) and it plays a great role in the interaction between phagocytes and ingested fungal cells (Wingard _et al_, 1982; Ray and Payne, 1988).

In the present investigation, the extrusion of extracellular protease from the isolates of the organism was detected qualitatively. The organism was inoculated in a suitable medium and its growth and enzyme activity was studied. The effect of different cultural parameters like temperature, pH and aeration (with or without the supply of
oxygen) were determined and an optimum condition was ascertained. Under these optimized conditions, the culture media were maintained for the maximum production of enzyme. These cultural conditions were further correlated with the growth of the organism.

Furthermore, from the optimized culture conditions, enzyme extracts were obtained and taken for the assay. The effects of different buffers like Citrate-Citrate, Citrate-Phosphate and Phosphate-Phosphate and Tris-HCl at their varying pH were used for the enzyme assay. Also concentration of various substrates (Casein, Egg albumin, Keratin, Gelatin), temperature, incubation time were determined in order to standardize the assay condition.

Section -1
Determination of the Extracellular Protease

MATeRIALS AND METHODS

Liquid Culture: The fungal isolates were grown in 150ml Erlenmeyer flasks containing 20ml of SD broth (Glucose, 40g; Peptone, 10g per litre at pH-5.6), were placed on the rotary shaker (110rpm) for 2 days. Under such condition, there will be a homogenous mixture of fungal cells. One ml of the culture solution was used to inoculate all experimental flasks to ensure uniformity of the inoculum.

Quantitative Test of Protease:

For the quantitative test of protease enzyme, a synthetic medium was selected with the following constituents: Starch, 1.0g; Casein powder, 10.0g; KH₂PO₄, 0.7g; K₂HPO₄, 0.3g; MgSO₄, 7H₂O, 0.5g and yeast extract 1.0g in 1 litre of distilled water. pH of the medium was adjusted to 6.0.

The isolates were grown in the above culture media. On completion of the desired growth i.e. after 11 days of incubation, the cells were separated from the medium by centrifugation at 10,000rpm for 20 minutes in cold condition (-8°C). The process was repeated to get rid of any cell debris. The supernatant was stored at -5°C to be used for enzyme assay.

The protease assay was performed according to the methods of Anson (1938) with slight modification as mentioned here. To 1.0ml of 1%(w/v) substrate solution, (Casein, being the most suitable one) in 0.1M citrate-phosphate buffer (pH-3.8), 0.2ml of enzyme solution (culture supernatant) was added. The reaction mixture was kept at 30±2°C for one hour, after which the activity was stopped by the addition of 1ml of
Fig. 1
Effect of Days of Incubation on Growth

Fig. 2
Effect of Days of Incubation on Protease Production

Growth in OD
Protease Activity in Units/ml.

Yeast Form
Mycelial Form
20% TCA. After a period of 20 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, 5.0 ml of 0.275 M Sodium carbonate, 2.0 ml of distilled water and 0.5 ml of 1:1 diluted Folin’s-Phenol-Ciocalteu reagent was added. The intensity of the blue colour developed after 20 minutes that was measured in Erma photoelecTris colorimeter using red filter (660 nm). All other conditions remaining same, a control was run in which TCA was added before the addition of the enzyme.

Protease activity units were subsequently calculated by matching the readings against a standard curve prepared by plotting colour values of tyrosine. 1 unit of proteolytic activity was considered as the amount of enzyme which liberated 1 μmol equivalent of tyrosine under experimental conditions.

**RESULTS**

Starting from the first day, growth of the isolates was taken turbidometrically on every alternate day. Protease assay was also performed on the same day. Equal pattern of growth and enzyme production was seen in both the forms; but the rate of growth in the yeast form was quicker and lesser as compared to the more faster mycelial form (Fig.: 1, 2). Growth of the isolates was maximum after 11 days of incubation, which declined sharply after subsequent days. Similar trend in protease production by the isolates of the organism was found as both the isolates showed maximum protease activity after 11 days of incubation.

**Section –II**

**Optimization of the Cultural Conditions**

**MATERIALS AND METHODS**

To detect the optimum growth and enzyme production, the isolates of the organism were grown in Sabouraud’s dextrose medium (Dextrose 40 g, Peptone 10 g in 1 litre of distilled water, pH adjusted to 5.6). The effect of different parameters like pH, temperature, incubation time and aeration on growth of the organism was studied and was correlated with the enzymatic activity.

1. **pH:** To determine the optimum pH for the maximum growth and extracellular proteolytic activity, 20 ml of the above mentioned medium were taken in each set of 150 ml Erlenmeyer flasks having pH adjusted to 2, 3, 4, 4.5, 5, 5.5, 6.0, 6.5, 7.0, 7.5, 8 and 9 respectively. In each set, two flasks were taken in excess for the stock cultures of each form of *C. albicans*. The cultures in the flasks were sterilized at 15 lb/inch² steam pressure for 20 minutes and kept undisturbed for
Fig. 3
Effect of pH on Growth

Fig. 4
Effect of pH on Protease Production

Growth in OD

Protease Activity in Units

Range of pH

Yeast Form Mycelial Form

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Yeast Form

Mycelial Form
24 hours. On the following day, the fungal inocula were transferred to the stock cultures with the desired pH.

II. **Temperature**: To determine the optimum temperature for growth and enzyme secretion of the isolates, the same media was prepared and stock cultures were also prepared as mentioned above. After incubation, the different sets of the flasks in triplicate were incubated at 15°C, 20°C, 25°C, 30°C, 33°C, 35°C, 37°C, 40°C, 45°C, 50°C.

1ml of the one day old inoculum from the respective stock cultures of each isolate was transferred aseptically to the flasks (in triplicate) and were incubated at 30±2°C. The incubation period was counted after twelve hours of inoculation. The optimum pH and temperature, for growth and enzyme activity of the isolates were obtained after 11 days of incubation.

III. **Effect of Oxygen**: The flasks containing 20ml of the Sabouraud’s dextrose media at the optimised pH and temperature were inoculated with the two indigenous isolates of *C. albicans*. One set of triplicate was incubated under shaking condition (in a rotary shaker of 110rpm) at room temperature to provide continuous supply of oxygen to the cultures. Another set was incubated at 30±2°C in the incubator in order to get the reading in static conditions.

The growth of the organism in both static and shaking cultures were taken turbidomTrisally on every alternate day after the 1st day of incubation. The EPR (extracellular protease) secretion was also calculated on the same day in Erma photoelecTris colorimeter using red filter (660nm).

**RESULTS**

The optimum pH for the growth and extracellular protease production of yeast and mycelial form was found in the pH range of 5.5 – 6.5. Yeast form grew best at pH 5.5 while the mycelial form had the best growth and activity at pH 6.5 (fig.: 3, 4). Similarly, when the isolates were grown in varying temperatures the yeast form grew well at 37°C, but the enzymatic activity was maximum at 30°C. (fig–5). A similar variation in growth and enzyme activity was observed by the mycelial form also. It had the peak growth at 33°C while it showed maximum activity at 30°C. (Fig–6).

The effect of oxygen in both the isolates was observed and it has been seen that the growth of the isolates in shaking culture was more rapid and extensive in comparison to static cultures. Similar results were obtained in the production of extracellular protease activity, which was much quicker, and faster then the static
Fig.: 5
Effect of Temperature on Growth

Protease Activity in Units

Fig.: 6
Effect of Temperature on Protease Production

Growth in OD

Protease Activity in Units

Yeast Form
Mycelial Form
Fig. 7: Effect of Oxygen on Growth

Days of Incubation: 1 to 15
Growth in OD

Fig. 8: Effect of Oxygen on Protease Production

Days of Incubation: 1 to 15
Protease Activity in Units

- Yeast Form
- Mycelial Form
cultures. Both the yeast and mycelial forms of the organism showed optimum growth and enzymatic activity after ninth day of incubation (Fig.-7, 8).

**Section-III**

**Standardization of Assay Conditions**

**MATERIALS AND METHODS**

The isolates of the organism were grown in a modified synthetic medium of Lilly and Barnett (Casein 10g; KH₂PO₄, 0.7g; K₂HPO₄, 0.3g; MgSO₄, 0.5g and yeast extract 1.0g per 1 litre of distilled water), in order to detect the optimum assay conditions, the effect of different parameters like pH (buffers), temperature, incubation time, substrate concentration and aeration were studied.

**Culture Medium:** To determine the optimum pH for maximum enzyme production, 20ml of the media were taken in each set of 150ml Erlenmeyer flasks having pH adjusted to 6.0. In each set, two flasks were taken in excess for the stock cultures of each form of *C. albicans*. The cultures were autoclaved at 121\textdegree\text{C}/inch² pressure for 20 minutes and kept undisturbed for 24 hours. On the following day, fungal inocula were transferred to the stock cultures. 1ml of 1 day old inoculum from the respective stock cultures of each isolates was transferred aseptically to the flasks (in triplicates) and were incubated at 30±2\textdegree\text{C}. The incubation period was counted after 12 hours of inoculation. The enzyme extracts were obtained as described earlier to know the assay characteristics.

**pH:** Different buffers with varying pH were used like citrate-citrate, citrate-phosphate and phosphate-phosphate Tris-HCl. To 1ml of 1% (w/v) substrate solution in 0.1M citrate-citrate/citrate-phosphate/phosphate-phosphate/Tris-HCl buffer (pH varying from 3.8-8.0) 0.2ml of enzyme solution (culture supernatant extracted by centrifugation) was added. The assay was performed at 30±2\textdegree\text{C} by the method described earlier in Section-I of this chapter.

**Temperature:** To determine the optimum assay temperature, the same above assay procedure was followed. To 1ml of 1% (w/v) substrate solution (Casein) 0.1M citrate-phosphate buffer (pH-3.8), 0.2ml of the culture supernatant were added. The mixture was then kept in incubator in varying temperature (20\textdegree\text{C}, 30\textdegree\text{C}, 33\textdegree\text{C}, 35\textdegree\text{C}, 37\textdegree\text{C}, 40\textdegree\text{C}, 50\textdegree\text{C}, 60\textdegree\text{C}) for 1 hour.

**Incubation Time:** To 1ml of the substrate solution with citrate-phosphate buffer (ph-3.8), 0.2ml of the enzyme extract was added. It was incubated at
Fig.: 9
Effect of Different Buffers on Protease Activity

Protease Activity in Units

Range of pH

Citrate - Citrate
- Yeast form
- Mycelial form

Phosphate - Phosphate
- Yeast form
- Mycelial form

Citrate - Phosphate
- Yeast form
- Mycelial form

This HCl
- Yeast form
- Mycelial form
Fig.: 10
Effect of Assay Temperature on Protease Production

Fig.: 11
Effect of Incubation time on Protease production

- Yeast Form
- Mycelial Form
Fig. 12
Effect of Substrate Concentration on Growth

Fig. 13
Effect of Substrate Concentration on Protease Activity

- ○ Yeast Form  – – Mycelial Form
Fig.: 14
Effect of different substrates on Protease Activity (Yeast Form)

Fig.: 15
Effect of different substrates on Protease Production (Mycelial Form)

- © - Gelatin
- • - Casein
- ▲ - Egg albumin
- ■ - Bovine serum albumin
30±2°C but the time interval for incubation was altered in the range of 20 minutes to 180 minutes. The assay was performed according to method of Anson as described in Section-I.

**Substrate Concentration:** To 1ml of the substrate solution, with citrate-phosphate buffer (pH-3.8), 0.2ml of the enzyme extract was added. Keeping all the parameters constant, the same procedure was followed by altering the concentration of the substrates (Casein, Bovine Serum albumin, egg albumin, gelatin) in buffer solution ranging from 0.25-2.5g per 100ml. It was incubated at 30±2°C for one hour. The assay was then performed as described in Section-I.

**RESULTS**

The fungal isolates grew well and their enzyme activity reached the peak in citrate-phosphate buffer at pH-3.8 (fig—9). The optimum assay temperature for the extracellular enzyme secretion by both the isolates was at 30±2°C (fig.—10). However, both the isolates showed optimum incubation time of one hour (fig.—11). Above or below this time, the enzyme activity of the organism declined. The optimum concentration of substrates for the assay condition was found to be 1% (Fig—12, 13). Casein being the most cheap, available and suitable one, was chosen though BSA showed equally good result. (Fig.—14, 15)

**DISCUSSION**

It has been observed that proteolytic enzymes are involved in many cellular and extracellular processes and they serve a variety of functions at the cellular tissue and systemic level. Under normal conditions, specific extracellular proteinases mediate the turnover of extracellular functions (Twinning, 1994). The death of cells within a tissue can result in the release of intracellular proteinases that causes degradation of the extracellular matrix. However, there are four major classes of endoproteinases based on the mechanism of catalysis and include serine, cystein (thiol), aspartic (acidic) and metalloproteinases. The *Candida* acid proteinase is a secreted extracellular aspartic proteinase as described earlier by Staib and colleagues (Staib, 1965; Remold *et al*, 1968). Since this extracellular proteinase (EPR) contain a higher percentage of aspartic acid residues, it is commonly called as aspartyl proteinase. However, *Candida* acid proteinase was originally designated as CAP (*Candida* aspartyl proteinase) and this group of proteinases has given a variety of labels. White *et al* (1993) suggested again that *Candida* secreted aspartyl proteinase may be referred to as SAP which is similar to original designation CAP. This enzyme is characterised as a carboxyl proteinase as it
has an acidic activity profile and is inhibited by Pepstatin A, an inhibitor of aspartic proteinases (Rüchel, 1981; Ray and Payne, 1990). To assess the role of aspartyl proteinase in cutaneous invasion, production of this enzyme in culture by several Candida species were assayed and correlated with their respective pathogenicities in experimental rodent models of cutaneous Candidasis and the results have shown that Candida acid proteinase participate in invasive cutaneous Candidiasis (Ray and Payne, 1990).

Keeping this fact in view, in the present investigation, assessment of the proteolytic activity has been done as a factor in the onset and development of Candida infections, here at Rourkela, Orissa.

Basically, micro-organisms have been classified on the basis of their pH range. Ingram et al (1993) classified them as acidophiles (pH 1.0 - 5.5), neutrophiles (pH 5.5-8.0) and alkinophiles (pH 8.5-11.5). However, both the isolates grew well in acidic pH though they could grow even in conditions of extreme acidic or alkaline range of pH. The yeast form of C.albicans grow best at pH 5.5 whereas the mycelial form had the peak growth at pH 6.5 (Fig.-3, 4). Since the best growth of the organism favoured an optimum pH range of 5.5-6.5; they were included in the border line of acidophiles.

A drastic variation in pH can harm the micro organisms by disrupting the plasma membrane or by inhibiting the enzyme activity and membrane transfer proteins (Olsen and Birkeland, 1977). The pH of the sweat secreted by the normal human body is slightly acidic. Blank (1939), Lang et al (1956), Olsen and Birkeland (1977) had of the opinion that there was a wide range of pH environment on the human body which can be affected by C.albicans. Das et al (1995) found that the humid conditions of Rourkela favoured excess secretion of sweat during the rainy season which in turn helped the organism in causing rapid mycotic superficial lesions during these months. Any change in the external pH could also alter the ionisation of nutrient molecules, reducing their availability to the micro-organisms (Ingram et al, 1993).

For standardisation, the isolates in the present study were assayed in different buffers having a varied range of pH. However, citrate phosphate buffer at pH 3.8 was the most suitable one where maximum proteinase activity of the organism was noted (Fig-9). The enzyme production gradually declined with the shift of assay pH towards the alkaline range. This finding was supported by various workers (Remold et al, 1968; Germaine and Tellefson, 1981; Hatori et al, 1984; Shimizu et al, 1987) and the phenomenon has been termed as alkaline denaturation. C.albicans can not grow at pH
6.0 or higher in buffered proteinase induction medium such as minimal broth, though it can grow at pH 3.7 in Sabouraud’s broth (Germaine and Tellefson, 1981; Matsuda, 1986). There was retardation in the growth and extracellular proteinase production at neutral or basic pH, which may be due to the absence of nitrogen supplementation by protein degradation. The optimum pH for enzyme production in certain species of \textit{C. albicans} was observed to be 3-4.5 with reduced activity up to 5.5 and the activity was irreversibly lost above 7.5 (Ray and Payne, 1990). In this investigation, there was feeble or negligible activity in higher pH and it almost diminished in pH range of 7-7.5. But even in alkaline pH, when the enzymatic activity was negligible, the growth was not found to stop completely. This suggests that isolates could grow better in medium having acidic range of pH. Since the pH of sweat is acidic and is secreted profusely from the human body (Stock-dale, 1953) under warm and humid conditions, the suitable environmental niche supported the organism to proliferate. But under unfavourable conditions (Odds, 1985) during the winter and summer months, the organism only survives as a commensal due to the alteration in the pH of human skin.

It is still unclear whether pH directly affects in the production of proteinase enzyme. The proteinase of \textit{C. albicans} so called acid proteinases, are of great importance and hence of special interest as this is related to the pathogenicity of the organism (Budtz-Jörgensen, 1971; Staib \textit{et al}, 1972; Germaine, 1978). Activity has been implicated in the attachment and penetration stage of \textit{Candida} infections (Borg and Ruchel, 1988; Ray and Payne, 1988, Ollert \textit{et al}, 1994) and correlation have been reported between pathogenicity and virulence of its strains (Rüchel, 1992).

In order to standardize the assay conditions, the medium composition was changed as many low molecular weights carbon and nitrogen sources causes suppression of proteinase in pathogenic fungi (Rüchel, 1986; Homma \textit{et al}, 1993). Hence it was changed adequately to determine the extracellular proteinase activity. However, in the present investigation, Sabouraud’s medium was not taken for the detection of proteinase enzyme in order to eliminate the chance of many soluble peptides and amino-acids in peptone besides 4% (w/v) glucose (Tsuboi \textit{et al}, 1985) inhibiting the extracellular enzyme production. Hence, starch was taken in place of glucose in the synthetic medium and amino acids were substituted by casein which serve as an effective inducer for the enzyme production rather than a nitrogen source.

BSA and few other proteins like gelatin, egg albumin were tested here as substrates in the culture medium in place of Casein. The effect of the nutrients on the
growth and enzyme production of the isolates were evaluated. The level of extracellular proteinase secretion varied depending on the inducer (Fig-14, 15). There was moderate enzymatic activity and growth in gelatin and egg albumen supplemented media by the isolates of the organism. Banerjee et al., (1991) had similar observations.

The optimum temperature for growth in both the variants of *C. albicans* in Sabouraud's dextrose medium was in the range of 30-37°C exactly at or below the humans body temperature. It has been observed that the temperature optima for the yeast form was 37°C where as for mycelial form it was 33°C (fig.-5). Again it is well stated that the dimorphic fungus which grow at the temperature range of 30-37°C are able to adapt the temperature of human body, i.e. 37°C (Gonzalez, 1984). However, the cells grown at room temperature can adhere to the epithelial tissues better than those grown at 37°C (Lee and King, 1983). From the above finding, it can be ascribed that, the yeast form of *C. albicans* which grow best at 37°C could have transformed itself into a pathogenic one when come in contact with the human host i.e. at 37°C, with a lag period required for incubation. On the contrary, the mycelial form that grow well at 33°C required no lag period and could cause mycotic lesions immediately when comes in contact with the host. This perhaps attribute to the greater pathogenicity of the mycelial form.

The growth temperature of *C. albicans* was found in the range of 20-40°C (Shephard et al., 1985; Odds, 1988; Anand and Prasad, 1991). Beyond this range, growth of the organism was observed to decline. Bezjak and Chandy (1989) had isolated the organism and grown in artificial media at 37°C. The maximum temperature for the growth of the organism was found in the range of 42-46°C by Doory et al. (1980) and Odds (1988). However, several reports suggested that the dimorphic yeast can die within minutes at temperature above 50°C (Stokes, 1971; Barreiro et al., 1981; Lemos Carlino and Madeira-Lopes, 1984; Madeira-Lopes and Cabecasilva, 1984). In the present study, similar results were obtained as both the isolates of the organism showed very little growth and enzyme activity at 45°C and almost died at 50°C. Several workers have suggested that (Dabrowa and Howard, 1984; Zeuthen and Howard, 1989; Howard et al., 1991) strains of *C. albicans* which grew at very high or low temperature secrete certain proteins called stress proteins which might have helped the organism to adapt to such extreme conditions.

Howard classified fungi based on the temperature of their optimum growth in the following order: Psychrophiles, temperature ranging from 0-17°C; Mesophiles from
15-40°C and Thermophiles ranging from 20-50°C. Here, both the isolates of *C. albicans* may be grouped under mesophiles as the temperature below 15°C and above 40°C were completely inhibitory. It has been suggested that temperature could exert a profound effect on aspects of growth, metabolism and survival of the yeast and the maximum growth temperature of the common yeast were in the range of 30-40°C (Stocks, 1971). There were no thermophilic yeasts which can grow above 50°C, although a few thermophilic filamentous fungi had been reported by Cooney and Emmerson (1964).

The optimum temperature is defined as the temperature at which the growth rate is highest and the overall biochemical process and associated enzymes involved in growth proceeds most rapidly or in other words, is the temperature which yields the largest cell crop. Farrel and Rose (1967) stated that at higher temperature, the enzyme and other cell formation were destroyed and impaired.

Here, it has been observed that the optimum temperature which favoured the maximum enzyme production for both the forms was 30±2°C. From the enzyme kinetics, it was observed that the proteinase activity was highest at the temperature range of 30-37°C at one hour of incubation time. However, in the present investigation, optimum temperature for maximum growth of the organism was found in the range of 33-37°C (when grown in Sabouraud’s dextrose broth). The organism being pathogenic requires the temperature same or below as the body temperature i.e. 37°C for the effective infection and colonization, on the human host. It has been reported that all epidermolytic proteinases have pH and temperature optima in the range of 4.0-4.5 and 35-37°C respectively (Maghrabi et al, 1990).

The isolates were observed under good supply of oxygen (shaking) and limited supply of oxygen (static) respectively. Both the strains of *C. albicans* showed good growth and enzyme activity under aerated condition than those grown on static conditions. Under static conditions, the cultures showed greater activity on 11th day of incubation, where as under shaking conditions, a comparatively higher results in the enzyme production was seen on the 9th day of incubation (fig.). The isolates showed uniformity in the increase of activity with increase of days of incubation. In static cultures, the growth and enzyme production was slower where as in shaking, it was comparatively faster. The proteinase production started earlier and consequent early decline in the activity in both the forms was observed in shaking cultures. Also, in shaking cultures, growth of the isolates was rapid in comparison to the static cultures and the growth and enzyme production run parallel to each other thus establishing a
direct link between the exponential growth of the organism with that of the enzyme production.

Several investigations have shown a limited degree of anaerobic growth of the organism (McClary, 1952; Samaranayake et al., 1983; Pollack and Hoshimoto, 1985). All isolates of the organism including *C. albicans* and excluding *C. guilliermondii* and *C. parapsilosis* showed exponential growth under anaerobic conditions (Webster and Odds, 1987). However, none of the isolates could grow under strict anaerobic conditions Anand and Prasad (1991) had of the opinion that oxygen might have a great influence on the respiration of the organism. Several electron transfer proteins are present in the mitochondria of the organism like NAD dehydrogenase, succinate dehydrogenase, cytochrome b, c and aa₃ (Mizuno and Montes, 1966; Yamaguchi et al., 1971; Hasilik and Livar, 1972; Borger et al., 1977; Shigematsu et al., 1982) TCA cycle enzymes were also found which provide protons and electrons to the cytochrome system (Odds, 1988). *C. albicans* also has an alternate oxidase (Chin et al., 1975; Kot et al., 1976; Shephard et al., 1978; Aoki and Itokawa, 1984) which is similar to the alternate cyanide resistant pathway (Lambowitz and Slayman, 1971; Downie and Garland, 1973; Laties, 1982; Guerin and Comougrand, 1986). These reasons may attribute to the growth of the fungal strains under conditions of limited supply of oxygen. Moreover, it has also been observed that *C. albicans* isolates are capable of growing under elevated concentrations of CO₂ in the air (Iralu, 1971; Webster and Odds, 1987). Infact for potentiating the dimorphic transition, a high CO₂ to O₂ ratio had been taken as an important factor in a large number of fungi (Szarisko et al. 1983). It has been found that when the skin was kept moist/wet continuously, the manifestation of Candidiasis increases (Frame et al., 1972; Aly and Maibach, 1983; Odds, 1988). The rate of CO₂ diffusion increases several folds under such coverings of bandages or wrist watch, which equilibrates with the underlying tissues in a few hours (Aly and Maibach, 1983). However, Eklund and Jarmud (1983) found that, an atmosphere of pure CO₂ is inhibitory to *C. albicans* growth. Again, it has been studied that the hyphal growth was associated with decreased activity of TCA acid cycle enzymes (Since less production of CO₂) repression of mitochondrial activity (reduced oxygen consumption) increase in cellular reduction potential and increase in the production of ethanol. All the above reasons simultaneously contribute to the great pathogenicity of mycelial form of the organism, here at Rourkela.