CHAPTER III

PHYSIOLOGICAL STUDIES
INTRODUCTION

Environmental factors have a definite effect on the organism, however, such effects account much on the sensitivity of the organism and its resistance to a particular factor. Amongst various factors temperature, relative humidity, available nutrients, radiations, pH of the growing medium etc. are the most important for the growth and development of micro-organisms.

Temperature affects the rate of all the processes occurring in micro-organisms and it may determine the type of reproduction, the morphology of the organisms and also their nutritional requirements. For any particular organism, four important temperatures may be defined. The thermal death point the minimum (10° - 15°C), optimum (24° -40°C) and maximum (35° - 45°C) temperatures. The minimum and maximum are the lowest and highest temperatures, respectively at which growth occurs and the optimum temperature is that at which the growth rate is the greatest. The minimum, optimum and maximum temperatures are known as the cardinal temperatures or points of the particular organism.

Light and darkness also affect some processes
of the micro-organisms. Light provides the energy for photosynthesis to autotrophic organisms; it causes oriented response in some organisms (tropic and tactic movements) or it may be required for or may stimulate sporulation in fungi and it can have deleterious or lethal effects.

Light responses are mediated by pigment which are transformed by absorption of light energy; this effect is translated into the observed response by light independent reactions (Nasim and James, 1978). For most non-photosynthetic organisms light is unnecessary or deleterious but in some fungi certain metabolic processes, including sporulation, are dependent on it. Sporulation of fungi both sexual and asexual is often initiated or increase by radiation in the ultra-violet or blue spectral regions. Thus zonation of a colony may occur when it grows in alternating periods of the light and dark bands of dense sporulation develop as a result of the periods of illumination. Light also influences pigment production in many fungi. Some normally pigmented fungi and mycobacteria fail to produce pigment or may affect the quantitative production of some pigments if grown in the dark.

There are many ways in which the hydrogen ion concentration or as it is usually expressed pH ($\text{pH} = -\log_{10} [\text{H}^+]$) of the environment may affect micro-
organisms. The net effect of pH acting on these various factors is expressed by the resulting growth and reproduction of the micro-organisms. The minimum pH for growth of most micro-organisms is generally about pH 2.5 and the maximum about 8-9; the optimum varies widely between and within the various groups of micro-organisms but is frequently between pH 5.0 and pH 7.5 (Ohta et al., 1975). Some imperfect fungi such as species of Penicillium and Aspergillus are capable of growing at the very low pH values of between pH 0.0 and 2.0 (Ohta et al., 1975). The pH range for reproduction of fungi is often reported less than that for growth.

Living cells are very well buffered internally against pH changes and environmental values have to be extreme before the intracellular pH is much affected. In extremely acidic or alkaline conditions the intracellular pH is much changed and intracellular enzymes may cease to operate. Extracellular enzymes are of course directly influenced by the environmental pH (Longworthy, 1978).

In pathogenic fungi, establishment of pathogen on host or its invasion in its body much depends on the external conditions, such as temperature, pH and various other factors including nutrient availability.
In the present chapter effect of different temperatures, light, darkness, and different pH is studied with a view to find out the most suitable conditions for the growth of otomycotic pathogens. Such studies may help in investigating the optimum conditions for establishment of these pathogens in external ear canal, which can be avoided in susceptible host by manipulation in the conditions of the external ear canal.
i - MYCELIAL GROWTH IN RELATION TO TEMPERATURES

MATERIALS AND METHODS

Five otomycotic pathogens i.e., Aspergillus niger, A. flavus, Absidia corymbefera, Penicillium nigricans and Candida albicans were used to determine the growth and temperature relationship. For this, Sabouraud's dextrose broth medium (Conant, et al., 1954) was used. Twenty five ml of culture medium was disposed in each 150 ml. erlenmeyer flask and the then autoclaved for 15 minutes at 15 lbs pressure. Flasks were then inoculated in duplicate by using one ml spore suspension prepared from the spores harvested from seven days old fungal colonies of test organisms. After inoculation flasks were incubated at 20, 30, 35, 40 and 45°C for seven days. After incubation mycelial content of each flask was harvested on preweighed filter papers (Whatman No. 1), washed with distilled water and dried at 60 - 70°C temperature till constant weight, and the average weight were recorded.

Before harvesting the mycelium tip of a needle was touched with spore and dipped in a cavity slide containing 0.05 ml sterilized distilled water and observed microscopically for sporulation.
RESULTS

Results of the present study are recorded in table no. 6. Most of the test fungi i.e., Penicillium nigricans (378 mg), A. niger (287 mg), A. flavus (272 mg) and A. corymbifera (208 mg) showed their maximum growth at 30°C. While in C. albicans maximum growth was recorded at 35°C showing a total of 143 mg dry mycelial weight.

All the test fungi have showed their growth at 45°C. Except C. albicans all the test fungi failed to sporulate when grown at 45°C temperature. C. albicans showed fair sporulation at this temperature.

All the fungi have showed their fairly good growth at 20°C but in most of the cases poor sporulation was revealed. A. niger showed good growth and sporulation at 25°C to 30°C while A. flavus showed its good growth at 25°C to 35°C. However, at 35°C this fungus produced less spores than grown at 25°C and 30°C.
TABLE NO. 6

Fungal growth and sporulation in relation to different temperatures

<table>
<thead>
<tr>
<th>Temperature</th>
<th>A. niger</th>
<th>A. flavus</th>
<th>A. corvus</th>
<th>P. nigricans</th>
<th>C. albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dry wt. (mg)</td>
<td>Sporulation</td>
<td>Dry wt. (mg)</td>
<td>Sporulation</td>
<td>Dry wt. (mg)</td>
</tr>
<tr>
<td>20°C</td>
<td>189</td>
<td>+</td>
<td>167</td>
<td>+</td>
<td>151</td>
</tr>
<tr>
<td>25°C</td>
<td>228</td>
<td>++</td>
<td>233</td>
<td>+++</td>
<td>162</td>
</tr>
<tr>
<td>30°C</td>
<td>287</td>
<td>+++</td>
<td>272</td>
<td>+++</td>
<td>208</td>
</tr>
<tr>
<td>35°C</td>
<td>280</td>
<td>+++</td>
<td>262</td>
<td>++</td>
<td>151</td>
</tr>
<tr>
<td>40°C</td>
<td>138</td>
<td>++</td>
<td>196</td>
<td>+</td>
<td>101</td>
</tr>
<tr>
<td>45°C</td>
<td>67</td>
<td>-</td>
<td>35</td>
<td>-</td>
<td>28</td>
</tr>
</tbody>
</table>

Poor = +
Fair = ++
Good = +++
No sporulation = -

Each datum given in table is an average of two independent determinations.
Fungal growth in relation to different temperatures

Mycelial growth (mg)

Test fungi:
A - A. niger
B - A. flavus
C - A. corymbifera
D - P. nigricans
E - C. albicans

FIG. 8
ii - SPORE GERMINATION IN RELATION TO TEMPERATURES

MATERIALS AND METHODS

The spore germination of *Aspergillus niger*, *A. flavus*, *Absidia corymbifera*, *Penicillium nigricans* and *Candida albicans* was determined at different temperatures i.e. 20, 25, 30, 35, 40 and 45°C. For this spore suspension of each test organism was prepared in sterilized Sabouraud's dextrose broth medium and the spores were allowed to germinate in the cavity slides kept in moist chambers at oforesaid temperatures. After 24 hours of incubation the spore suspension from each cavity slide was examined under low/high power of the microscope. More than two hundred spores were counted in each case and percentage of germinated spores was calculated.
RESULTS

After 24 hours germination of spores of *A. corymbifera, A. niger, A. flavus* and *P. nigricans* was recorded at 30°C. *C. albicans* showed (Table - 7) maximum spore germination at 35°C. At high temperature i.e., 45°C this fungus showed the germination of 19.50 percent spores only. While in all other cases not more then 7.50 percent spores were found to germinate at this temperature.

Spore germination in *C. albicans* at 20°C was recorded quite less i.e., only 3.00 percent while other fungi showed more than 20 percent germination of spores at this temperature.
TABLE NO. 7

Inhibition (%) of fungal spore germination at different temperatures after 24 hours of incubation.

<table>
<thead>
<tr>
<th>Temperature used</th>
<th>A. <em>niger</em></th>
<th>A. <em>flavus</em></th>
<th>A. <em>corynebacterium</em></th>
<th>P. <em>nigricans</em></th>
<th>C. <em>albicans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>20°C</td>
<td>31.00</td>
<td>25.00</td>
<td>25.50</td>
<td>21.00</td>
<td>03.00</td>
</tr>
<tr>
<td>25°C</td>
<td>55.00</td>
<td>44.50</td>
<td>65.00</td>
<td>45.00</td>
<td>29.00</td>
</tr>
<tr>
<td>30°C</td>
<td>85.00</td>
<td>78.50</td>
<td>92.50</td>
<td>67.50</td>
<td>58.00</td>
</tr>
<tr>
<td>35°C</td>
<td>53.00</td>
<td>39.00</td>
<td>42.00</td>
<td>51.00</td>
<td>79.50</td>
</tr>
<tr>
<td>40°C</td>
<td>26.00</td>
<td>27.00</td>
<td>19.50</td>
<td>14.00</td>
<td>32.50</td>
</tr>
<tr>
<td>45°C</td>
<td>06.50</td>
<td>02.50</td>
<td>07.00</td>
<td>04.50</td>
<td>19.50</td>
</tr>
</tbody>
</table>

For each observation more than 200 spores were counted.
INHIBITION (%) OF FUNGAL SPORE GERMINATION OF DIFFERENT TEMPERATURES

- A. niger
- A. flavus
- A. corymbifera
- P. nigricans
- C. albicans

FIG. 9
iii - SPORE GERMINATION IN RELATION TO DIFFERENT INCUBATION PERIODS

MATERIALS AND METHODS

To study the effect of light and darkness on the spore germination, five test fungi i.e., A. niger, A. flavus, A. corymbefera, P. nigricans and C. albicans were used. The effect of different hours of light / darkness on spore germination was determined. Spore suspension of each test organism was prepared in sterilized Sabouraud's dextrose broth medium. 0.05 ml of suspension of each type of test spores was placed in cavity slides with the help of sterilized micro - pipettes. Spore suspension were then given different periods of light and darkness. The period of light and dark exposures are as follows:

(1) 24 hours light
(2) 18 hours light / 6 hours darkness
(3) 12 hours light / 12 hours darkness
(4) 6 hours light / 18 hours darkness
(5) 24 hours darkness.

For light treatments 40 watt. bulb was used as a source of light. The treated spore suspension were incubated at 28°C during treatment.
After treatment a number of spores which showed germination were counted. In each case at least 200 spores were counted and percentage of germinated spores was calculated.
RESULTS

The results showing the effect of periods of lightness and darkness on spore germination are given in table 8. The data reveals that maximum germination of spores in *A. corymbifera* (81.00%) was recorded when spores were exposed to 18 hours light and 6 hours period of darkness, while in all other test fungi only 30-41 percent spore germination was recorded during above period of light and dark treatment. A throughout dark period of 24 hours supported the spore germination in most of the test fungi in comparison to complete light period (24 hours).

Twelve hours light followed by 12 hours darkness supported maximum spore germination, while in case of *A. corymbifera* maximum spore germination was obtained when spores were exposed to 18 hours light and 6 hours dark period. In case of *A. niger* and *A. flavus* 6 hours light followed by 18 hours dark treatment induced maximum spore germination. In these cases 65 and 69 percent spore germination was recorded.
### TABLE NO. 8

Spore germination (%) in different conditions of incubation

<table>
<thead>
<tr>
<th>Incubation condition in hours</th>
<th>A. niger</th>
<th>A. flavus</th>
<th>A. corvusfera</th>
<th>P. nigricans</th>
<th>C. albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h Light</td>
<td>19</td>
<td>16</td>
<td>33</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>18h / 6 h Light/ Dark</td>
<td>30</td>
<td>32</td>
<td>81</td>
<td>30</td>
<td>41</td>
</tr>
<tr>
<td>12h / 12 h Light/ Dark</td>
<td>41</td>
<td>50</td>
<td>72</td>
<td>62</td>
<td>70</td>
</tr>
<tr>
<td>6h / 18 h Light/ Dark</td>
<td>65</td>
<td>69</td>
<td>61</td>
<td>51</td>
<td>67</td>
</tr>
<tr>
<td>24h Dark</td>
<td>59</td>
<td>63</td>
<td>41</td>
<td>47</td>
<td>52</td>
</tr>
</tbody>
</table>

\[ h = \text{hours} \]

For each observation more than 200 spores were counted
SPORE GERMINATION PERCENTAGE IN DIFFERENT CONDITIONS OF INCUBATION

- ○ A. niger
- △ A. flavus
- □ A. corymbifer</ref>
- ★ P. nigricans
- ■ C. albicans

PERCENTAGE OF SPORE GERMINATION

LIGHT/DARK

HL = HOURS LIGHT
HD = HOURS DARK

FIG. 10
iv - EFFECT OF DIFFERENT pH ON SPORE GERMINATION

MATERIALS AND METHODS

The effect of hydrogen -ion concentration was studied on spore germination of five test fungi i.e., Aspergillus niger, A. flavus, Absidia corymbifera, Penicillium nigricans and Candida albicans. For this pH 4, 5, 6, 7, 8 and 9 were selected. Spore suspension of each test organism was prepared in Potassium phosphate buffer of different pH. So prepared buffers were than autoclaved and spore suspension of equal turbidity were prepared. 0.05 ml spore suspension of each test fungus was than transferred in cavity slides and incubated at 28°C. T. 12330

After 24 hours of incubation the spore suspension from each cavity slide was examined and percentage of spore germination in each case was noted. Two hundred spores were counted in each case and percentage of germinated spores was calculated.
RESULTS

The data presented in table 9 indicate that maximum germination of spores in *A. corymbefera* (84.00%), *A. niger* (79.00%), *A. flavus* (63.00%) and *P. nigricans* (59.00%) was recorded in 24 hours at pH 5.0. While maximum spore germination in *C. albicans* (62%) was noted at 6.00 pH. In the case of *C. albicans* 23.00 percent spore germination was recorded at pH 5.00.

At pH 7.0 spore germination in *A. flavus*, *A. niger*, *A. corymbefera* and *P. nigricans* recorded was 29.00, 23.00, 21.00 and 14.00 percent, respectively. At a low pH (4.0) 43.00, 38.00, 35.00, 11.00 and 3.00 percent spore germination was recorded in *A. flavus*, *A. niger*, *P. nigricans*, *A. corymbefera* and *C. albicans*, respectively. However, at pH 9.0 lesser number of spores were found to germinate. At pH 9.0 in none of the cases spore germination exceeded to 50 percent.
TABLE NO.9

Spore germination (%) in different pH

<table>
<thead>
<tr>
<th>pH</th>
<th>A. niger</th>
<th>A. flavus</th>
<th>A. corymbifer</th>
<th>P. nigricans</th>
<th>C. albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.00</td>
<td>38</td>
<td>43</td>
<td>11</td>
<td>35</td>
<td>3</td>
</tr>
<tr>
<td>5.00</td>
<td>79</td>
<td>63</td>
<td>84</td>
<td>59</td>
<td>23</td>
</tr>
<tr>
<td>6.00</td>
<td>42</td>
<td>47</td>
<td>36</td>
<td>21</td>
<td>62</td>
</tr>
<tr>
<td>7.00</td>
<td>23</td>
<td>29</td>
<td>21</td>
<td>14</td>
<td>38</td>
</tr>
<tr>
<td>8.00</td>
<td>17</td>
<td>8</td>
<td>13</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>9.00</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

For each observation more than 200 spores were counted

Time of incubation = 24 hours
SPORE GERMINATION PERCENTAGE IN DIFFERENT pH

- A. niger
- A. flavus
- A. corymbifera
- P. nigricans
- C. albicans

**FIG. 11**
DISCUSSION

A perusal of the tables 6 and 7 indicates that most of the test fungi showed maximum mycelial growth and spore germination at 30°C but C. albicans showed its best growth at 35°C. In almost all the cases decreased growth of almost all the test organisms was noted at 45°C. Since an important part of the growth process is conceived to be operated by enzymatic systems of an organism and their enzymatic reactions are regulated by temperature, probably higher temperature is affecting the enzymatic system in test organisms and hence, a decrease in the mycelial growth and sporulation was noted.

In the present study the suitable temperature range for satisfactory growth in all the test fungi except C. albicans determined was between 25 to 35°C while the optimum temperature for mycelial growth and spore germination of four of the test fungi is determined to be 30°C. Temperature can also affect other activities of micro-organisms quite apart from its effect on growth. Production of pigments by some organisms is affected by temperature. Fabricant and Perislain (1949), has shown that the temperature of the external ear canal remains between 29.4 °C and 34.1 °C with an average of 31.5 °C. It is
generally believed that a large number of otomycosis cases occur in hot and humid climatic conditions. Otomycosis is rarely observed in cool and dry climates (Singer et al., 1952).

During the study of the effect of light and dark incubation periods on spore germination, maximum spore germination in different test fungi was found different. *P. nigricans* and *C. albicans* showed maximum spore germintion when spores of these organisms were exposed to 12 hours light and 12 hours dark period. While in other fungi spore germination was not recorded maximum in above light and dark incubation. *A. flavus* and *A. niger* showed maximum germination of spores in 6 hours light followed by 18 hours darkness.

pH of the medium is one of the important factors for the growth and sporulation of almost all the micro-organisms. In present studies pH range for satisfactory spore germination was also found to be different for different test fungi. For all except *C. albicans* pH 5.0 was found to be the optimum for spore germination. Yassin et al., 1964 have examined the growth of three otomycotic pathogens and reported maximum growth of *A. terreus*, *A. flavus* and *A. niger* at pH 5.0. Results of the present study on spore germination supports the
findings of above worker.

Living cells are very well buffered internally against pH changes but inextreme environmental pH values, intercellular pH is also gets affected. Most micro-organisms produce extracellular enzymes which modify the substrates in utiliseable forms which upon hydrolysis are observed and hence, other metabolic functions get triggered inside the cell. Extracellular enzymes, hence directly affect the extracellular enzymes and their activity. Such a change in pH directed changes in enzymes and finally results in the non availability of the nutrients for proper growth and development of fungi.

In pathogenesis pH of the external media plays an important role as the microbes have to invade the host cell membrane for its establishment and further growth. In present studies pH 8.0 - 9.0 was not found to be suitable for the spore germination in most of the test fungi. Yassin et al., 1964 also reported this and suggest that if the pH of the external ear canal can be buffered to pH 9.0 by some means the establishment of fungal infection can be avoided.